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THE

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EFFECT OF UREA, THIOUREA, PHENYLTHIOUREA AND THIOURACIL ON THE OXYGEN CONSUMPTION OF BLOCKED AND ACTIVE EMBRYONIC CELLS [1]

JOSEPH HALL BODINE AND LAURENCE ROCKWELL FITZGERALD

Zoological Laboratory, State University of Iowa, Iowa City, Iowa

INTRODUCTION

Considerable data on the action of urea, thiourea, thiouracil and related compounds on the intact organism are at hand, especially as regards their action in such phenomena as growth and thyroid function (Lynn, 1948). That thiourea and thiouracil are important in the activity of the thyroid gland has been widely demonstrated both clinically and otherwise. The exact mechanism of their action on cells, however, seems to be less well understood. The fact that certain oxidations, involving cytochrome-cytochrome oxidase or the peroxidase systems, are significant in the reactions of thiourea and thiouracil seems well established (Sadhu, 1948). It becomes of some interest, therefore, to compare the effects of such chemicals upon the respiratory mechanisms of embryonic cells, both in the mitotically active as well as in the resting or blocked condition. The present paper has to do with results of studies on the effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of mitotically active and blocked cells of the embryo of the grasshopper, *Melanoplus differentialis.*

MATERIALS AND METHODS

Eggs of the grasshopper, *Melanoplus differentialis*, were obtained and dissection of embryos carried out as previously noted (Bodine and Boell, 1934). Sterile phosphate-buffered Ringer solution (pH 6.8) was used as the suspending medium for the embryos. All solutions of chemicals (c.p.—urea, thiourea, phenylthiourea, thiouracil [2]) were made up in this buffered Ringer solution. Diapause (blocked) and post-diapause (active) embryos were, from all external appearances, morphologically identical (Bodine and Boell, 1936). Only embryos from eggs

[1] Aided by grant from the National Institute of Health.

[2] Samples of thiouracil were generously donated by the Lederle Laboratory of Pearl River, N. Y.

of known temperature and developmental histories were employed. Diapause eggs were those kept constantly at 25° C. from the time of laying, and which were then confirmed by O_2 uptake determination to be in the blocked state. Post-diapause eggs were those diapause eggs previously kept at 5° C. for periods long enough to remove the block and which were also confirmed by further O_2 consumption tests to be in the active state.

Oxygen determinations were carried out at 25° C. with standard Warburg equipment, using 5 cc. flasks. Control or blank runs on all embryos were for 30 minutes, after which the reagents were added from the side arms and the oxygen consumption rates followed for periods of no less than one hour. Three banks of manometers, 18 in number, were used in individual runs and general averages calculated. Each concentration of reagent was tested many times, so that over-all reading totals for each point represent several hundred determinations. One hundred embryos were used in each flask. In comparing reactions of blocked and active embryos, all determinations on a single lot were carried out simultaneously, so that experimental conditions were kept similar throughout.

Concentrations of reagents were calculated from the quantities added to the vessels and it was found that the degree of effect shown by 50 embryos compared favorably with that for 100 embryos, indicating that no appreciable amounts of the reagents were taken up by the embryos.

Results

Urea

The results of the effects of urea upon the oxygen uptake of both active and blocked cells of embryos are graphically depicted in Figure 1. An inspection of

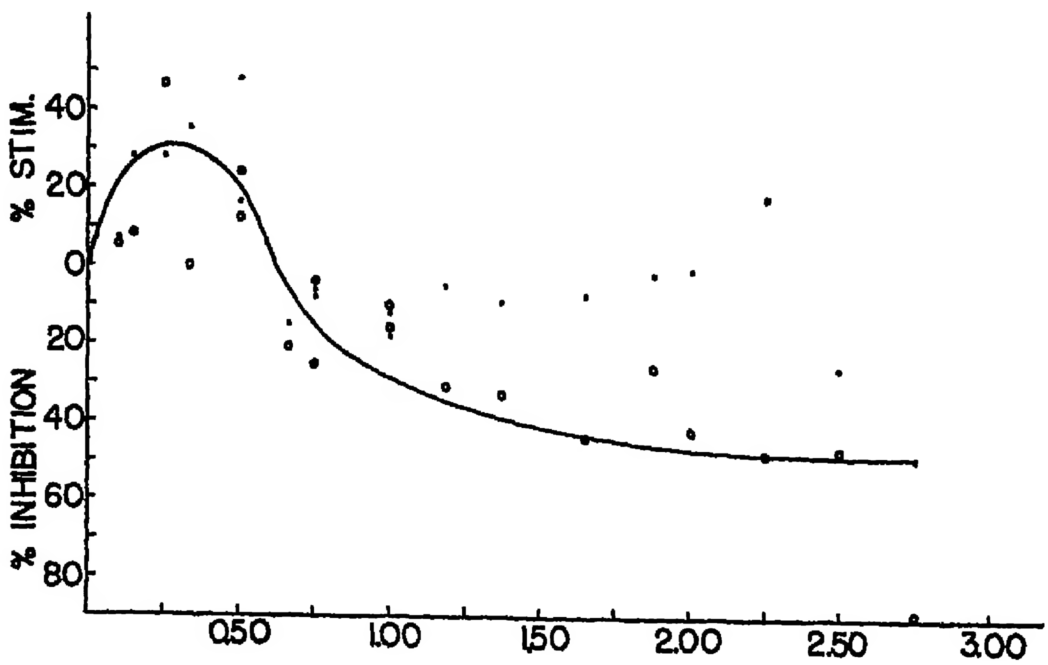

Figure 1. Effect of concentration of urea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of urea. Solid curve is average for all experiments on blocked and active embryos for low concentrations and for active embryos in high concentrations. Results for a typical experiment are indicated—solid circles for blocked, open circles for active embryos.

this figure shows a marked stimulation of the oxygen consumption with low doses of urea and a corresponding inhibition with higher doses. The general shape of the curve is similar to that found for ethyl carbamate (Bodine and Fitzgerald, 1948), and is perhaps more or less typical of the reaction of this material to many such reagents. No significant differences in the response of blocked and active cells are apparent for the stimulating effects of low concentrations of urea. For higher, inhibitory doses, however, a marked resistance to the reagent is shown by the blocked cells, the mitotically active cells being much more affected by such exposures. Up to concentrations of approximately 2 molar, the inhibitory effects of the urea on the oxygen uptake of blocked cells are small and completely reversible. For the active cells at concentrations of 1.25 molar, one gets about 50 per cent recovery in oxygen consumption rates, while at 2 5 molar the maximum average recovery amounts to but 25 per cent.

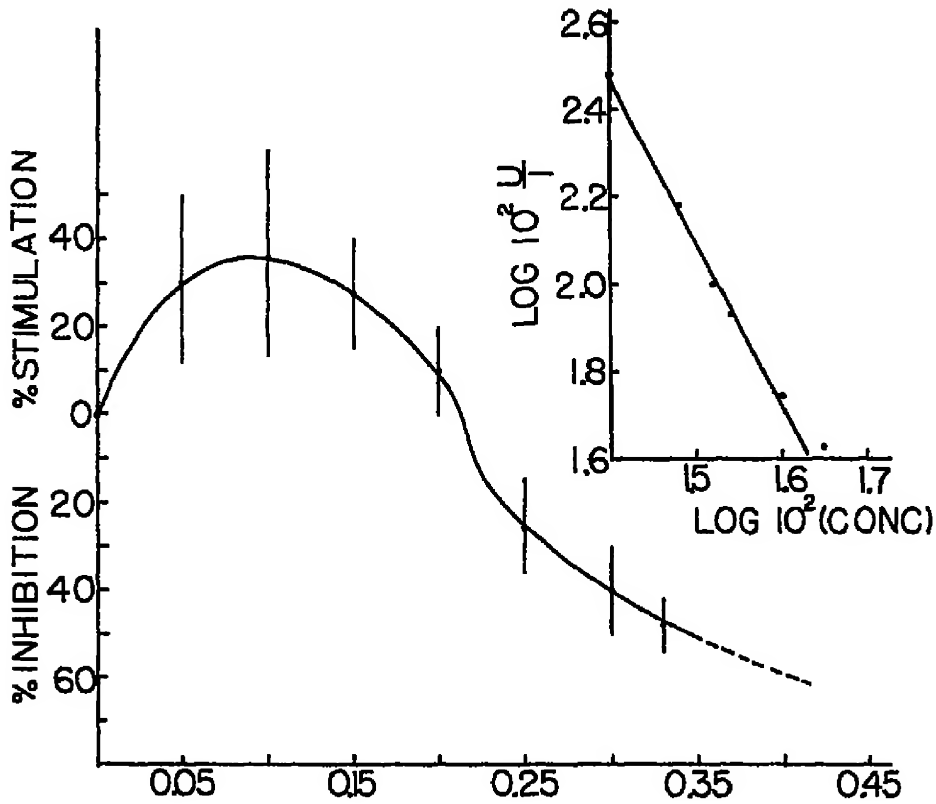

FIGURE 2 Lower left, effect of concentration of thiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of thiourea. Solid curve is average for all experiments. Vertical lines through points represent extent of variation for specific concentrations. Upper right, a log-log plot showing relation between ratio of uninhibited (U) and inhibited (I) respiration and concentration of thiourea. Ordinate, log 10^2 U/I. Abscissa, log 10^2 concentration of thiourea.

A point of some interest as regards the reaction of the embryos, both blocked and active, to urea is that after exposure to the reagent and subsequent washing and suspension in Ringer solution, a decided swelling is observed. The intensity of this swelling seems dependent upon the concentration of urea employed. Such a reaction has been observed only after exposure to urea and no other compound. Further details concerning this reaction will be dealt with in a subsequent report.

Thiourea

The effects of thiourea on the oxygen consumption of cells are qualitatively similar to those found for urea. Results are shown graphically in Figure 2. Marked stimulation of oxygen uptake by low concentrations and inhibition by higher concentrations are invariably produced. No significant differences in response to any concentration of the thiourea by the blocked and active cells are noted. Practically complete recovery in oxygen uptake occurs, except for the highest concentrations of reagent employed. The relative effective concentrations of thiourea, as will be noted from Figure 2, are much lower than for urea.

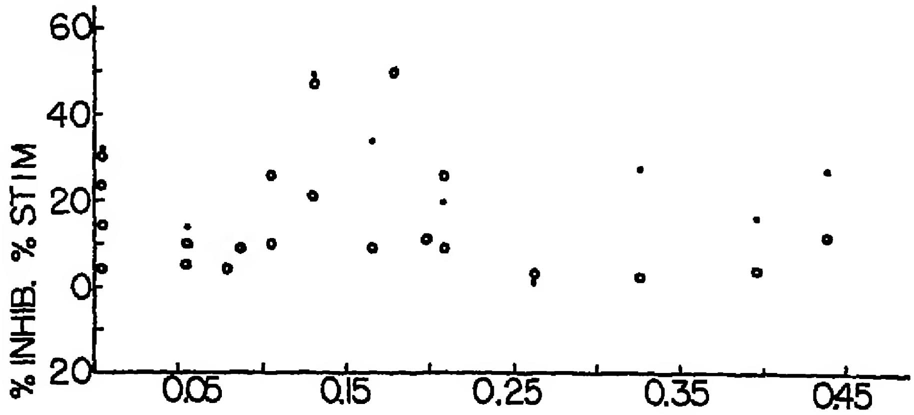

FIGURE 3. Effect of concentration of phenylthiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration $\times 10^3$ of phenylthiourea. Results for typical experiments indicated. Solid circles for blocked, open circles for active embryos.

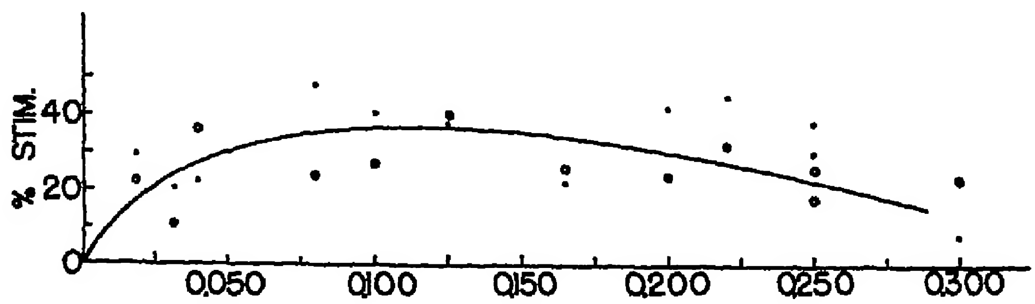

FIGURE 4. Same as Figure 3, but for thiouracil. Solid curve is average for all experiments. Results for typical experiment shown.

Phenylthiourea

The action of phenylthiourea on the oxygen uptake of both blocked and active cells is quite similar to that for urea and thiourea. As indicated in Figure 3, only stimulation of the oxygen uptake has been found for the concentrations employed. Such a result is doubtless due to its low solubility and to the low concentration of the compound used. Complete recovery is found for all concentrations of the reagent.

Thiouracil

Thiouracil, like phenylthiourea, is but slightly soluble and hence only low concentrations of this reagent are available. A marked increase in oxygen uptake

for both blocked and active cells is found (Fig. 4). No significant differences in response for blocked or active cells are noted. When washed and resuspended in Ringer solution, complete recovery in oxygen uptake occurs.

Discussion of Results

The rather marked and similar effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of both blocked and active embryonic cells are indeed striking, and several points of general interest in respect to their fundamental action arise. The fact that thiourea and thiouracil have been shown to be involved in the respiratory mechanisms of cells (Sadhu, 1948) would lead one to suspect similar actions on the oxygen uptake mechanisms of the type of cells employed in these investigations.

The oxygen consumption rates of blocked and active embryos (those mitotically blocked or active) are markedly different (Bodine and Boell, 1934). For morphologically similar embryos, the rates of oxygen uptake of active embryos are at least three to four times that of blocked ones. It seems important, therefore, in any discussion of the effects of various reagents upon the oxygen consumption of these forms, to keep in mind these basic differences in rates of oxygen uptake associated with their cellular behavior. Urea is the only compound found to differentially affect the oxygen consumption of the embryos, and then in such high concentrations as to be rather toxic for the active ones. Blocked cells are but little affected by relatively high concentrations of urea, and then in a completely reversible manner. For all other compounds employed, no significant differences in the response of blocked and active cells are found. Any consideration of the effects of these compounds, therefore, will refer equally to both blocked and active cells.

The characteristic curve of response to different concentrations of these reagents—a marked stimulation of oxygen uptake in low concentrations and a similarly marked inhibition in higher concentrations—compares favorably with results found for ethyl carbamate (urethane) and related compounds (Bodine and Fitzgerald, 1948). Urethanes are thought to have marked effects upon the dehydrogenases or carriers of the respiratory mechanisms of cells. Thiourea and thiouracil inhibit certain enzyme systems, probably the oxidase and peroxidase activity of cells (Sadhu, 1948).

Many invertebrates, as is well known, use copper in functions normally taken over in higher forms by iron, for example haemocyanin. Grasshopper embryos, at the stages used in the present work, contain appreciable amounts of copper (approximately 0.025 μgm. per embryo [3]), and it is reasonable to assume that it functions for them in enzyme systems in a manner comparable to other cations for higher forms. It is also well known that copper has marked action upon many enzyme systems, and especially sulfhydryl-containing enzymes (Barron and Singer, 1945). As a specific example, it has been rather clearly shown that copper markedly inhibits the hemolysis of red blood cells in isosmotic glycerol solutions (Jacobs and Carson, 1934). Explanations of such phenomena suggest the action of copper on a sulfhydryl-containing enzyme system located in the red blood cell (LeFevre, 1948). It becomes of some interest, therefore, to examine the possibility of the part played by the contained copper of grasshopper embryos in their reactions to the reagents employed.

It can readily be shown that tyrosinase, a copper-containing enzyme, isolated from the grasshopper egg, is markedly inhibited by thiourea.[4] Such a reaction for this enzyme is well known, and the explanations based upon a copper-thiourea reaction seem well founded (Denny, 1943). The possibility of such copper combinations with the other reagents employed seems worthy of comment.

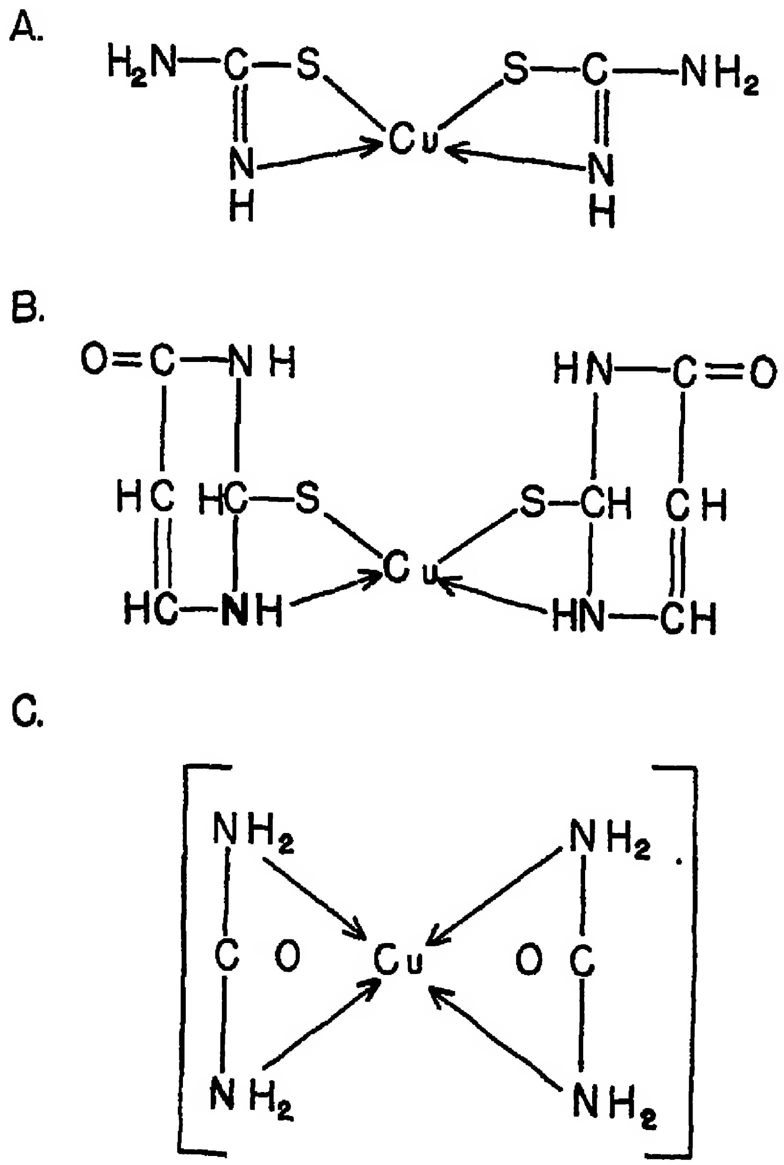

FIGURE 5. Scheme for possible combinations of reagents with copper. A = thiourea, B = thiouracil, C = urea.

For thiourea and thiouracil (A and B of Fig. 5[5]), there is some evidence for the existence of the copper combination as indicated. Theoretically, there is no reason for which a complex copper-urea ion could not exist for urea (C of Fig. 5) in a form essentially as shown. Recently it has been pointed out by LeFevre (1948) that the molecular concentration of reactivating substances (containing SH groups) had to equal, or exceed by one to two times, the concentration of the inhibitor (copper) used in the case of hemolysis of red blood cells. Experiments carried out in

[4] Unpublished data from this laboratory.

[5] Acknowledgment is made to Professors George Glockler and Ralph L. Schriner of the Department of Chemistry for their suggestions in this problem.

connection with the present investigations, using concentration of copper and of thiourea in such proportions, corroborate the findings of LeFevre.

The effects of thiourea and thiouracil on cell respiration and cell division have been variously reported; in some cases inhibition, while in others stimulating responses, are described (Fearon, 1942). Embryos of the grasshopper while in the blocked condition have been shown to lack mitotic activity and to have rates of oxygen uptake at a low constant level. When active, mitosis is always present and rates of oxygen uptake are increasingly higher than for the blocked or inactive states. Such naturally-occurring cellular conditions, therefore, make possible checks on the parts they play in reactions to various reagents. Urea is the only compound with which a marked and significant difference in response between blocked and active cells is noted. Blocked cells are extremely resistant to this compound while active ones are markedly affected, and usually in an irreversible manner. No explanation for such a basic difference in response is at hand.

Published data concerning the effects of urea, etc., on the respiration of cells are fragmentary or almost completely lacking, so that comparisons between copper-containing cells like those used in the present experiments, and those for higher forms, seem practically impossible. The bacteriostatic action of urea alone and in combinations with the sulfonamides seems well established (Kirby, 1943). Alkyl ureas have been shown to depress certain respiratory enzymes (dehydrogenases, coenzymes) (Grant and Krantz, 1942). The effects of thiourea as well as of thiouracil upon such basic phenomena as mitosis, however, seem at present rather confused (Paschkis, Cantarow, Rakoff and Rothenberg, 1945). The effects of thiourea and thiouracil upon the growth of amphibians, as well as upon the thyroid gland itself, are rather clear and well defined (Lynn, 1948).

The stimulating effects of low concentrations of all reagents, although typical, are for the present not readily explained. Permeability and other factors have been suggested for similar phenomena in other forms (Lillie, 1916). Lack of inhibition of oxygen uptake in the case of phenylthiourea and thiouracil appears to be the result of the low solubility of these compounds, and thus for them solubility becomes the limiting factor.

The most regular and consistent responses for any reagent used are those for thiourea, and even here solubility of the compound becomes the limiting factor in a complete analysis of its effect on oxygen consumption. Responses to it for blocked and active cells are not significantly different. An analysis of its inhibitory effects according to the law of mass action, as pointed out by Fisher and others (Fisher and Henry, 1943), would suggest that its action may be similar to that for urethane and that a single respiratory mechanism may be involved (Fig. 2).

Summary and Conclusions

1. The action of urea, thiourea, phenylthiourea and thiouracil on the oxygen uptake of the blocked and active cells of the embryo of the grasshopper, *Melanoplus differentialis*, has been studied.

2. In general, low concentrations of these reagents produce stimulation, while higher concentrations produce inhibition of the oxygen consumption of both blocked and active cells.

3. Urea alone produces a differential effect, in that blocked embryos are little affected by high concentrations while active embryos are irreversibly inhibited.

4. The relative effectiveness of the compounds upon the oxygen consumption of blocked and active cells is: thiouracil > phenylthiourea > thiourea > urea; recovery seems to work in the opposite order.

5. Suggestions are made as to the possible significance of copper in determining the basic reaction of these compounds.

ACKNOWLEDGMENT

Acknowledgment is gratefully made to Etta Andrews, John Johnston, and Herman Tharp for technical assistance in carrying out these experiments.

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THE EFFECTS OF ELECTROLYTES AND SUGARS ON THE ERYTHROCYTES OF THE TURTLE, CHELYDRA SERPENTINA[1]

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The erythrocytes of the snapping turtle, *Chelydra serpentina,* exhibit a particular sensitivity to the lack of calcium and will hemolyze in electrolyte solutions which do not contain this ion, though not in pure glucose (Lyman, 1945). The absence of hemolysis reported for glucose is presumed to be due to impermeability to the glucose molecule because of its size. If this is true, sugars of smaller molecular volume might prove hemolytic. Two factors other than size may also have an influence. From Gibbs-Donnan equilibrium relationships, changes in membrane structure may be expected when a non-electrolyte replaces an electrolyte medium, all sugars having an equivalent action in this respect. In addition, sugars might affect the cell membrane by direct chemical action, and such effects may be characteristic for the individual sugars. With regard to the hemolytic action of calcium-free electrolyte solutions on Chelydra erythrocytes, it is not known to what extent electrolytes differ in their action, nor in what manner an alteration of the hydrogen ion concentration of any given solution may influence the hemolytic process. These various factors have been considered in the present study.

The results show that hemolysis occurring in calcium-free electrolyte solutions is influenced by the ionic composition of the medium. Moreover, by suitable adjustment of the hydrogen ion concentration, the integrity of the cell membrane may be maintained for short periods even in the absence of calcium. In an examination of the effects of isosmotic solutions of various sugars, striking differences were found, some being hemolytic and others not. Agglutination and hemolytic reactions observed in certain sugars indicate that these compounds are not inert, but produce a definite alteration of the cell membrane.

Material and Methods

Blood (0.5 – 1.0 ml.) of *C. serpentina* drawn without anticoagulant was washed twice in 40 ml. of frog Ringer and suspended in Ringer. In determining the rate of hemolysis, washed cells were centrifuged briefly; the supernate was very carefully removed, and 5.0 ml. of experimental solution added to give a cell concentration of approximately 1 : 100. The optical density was measured within the first minute and at intervals with a Fisher electrophotometer (Wilbur and Collier, 1943). A Beckman spectrophotometer, made available through the kindness of Dr. W. J. Dann, was used for determinations of the optical density of hemoglobin solutions and for the hemoglobin spectrum of turtle blood.

[1] Aided by a grant from the Duke University Research Council.

Hematocrit tests showed frog Ringer to be approximately isotonic with slight variations between individuals. Experimental solutions were made isosmotic with 0.125 molal NaCl. Double distilled water was used throughout. Galactose, xylose and arabinose were Pfanstiehl brand and were free of calcium. NaCl was Merck Reagent For Biological Work. Other chemicals were reagent grade and were not further purified.

We wish to thank Dr. M. H. Jacobs and Dr. H. B. Collier for their helpful suggestions and Mr. N. G. Anderson and Mr. R. L. Rigsbee for photographic work.

Results

Electrolytes

The observation of Lyman (1945) that erythrocytes of *C. serpentina* will hemolyze in isotonic Ca-free salt solutions was readily confirmed. Moreover the course of hemolysis in isosmotic NaCl, for example, could be arrested by the addition of a small amount of isosmotic $CaCl_2$ solution to the hemolyzing suspension. However, in preliminary experiments it became apparent that the rate of hemolysis varies with the cation and anion employed and also with the hydrogen ion concentration.

The effect of different cations was examined by following the course of hemolysis in buffered and unbuffered isosmotic solutions of NaCl, KCl, $MgCl_2$ and $CaCl_2$. Hemolysis was always most rapid in KCl, followed by NaCl, slower in $MgCl_2$ (Figs. 1 and 2), and completely absent in $CaCl_2$.

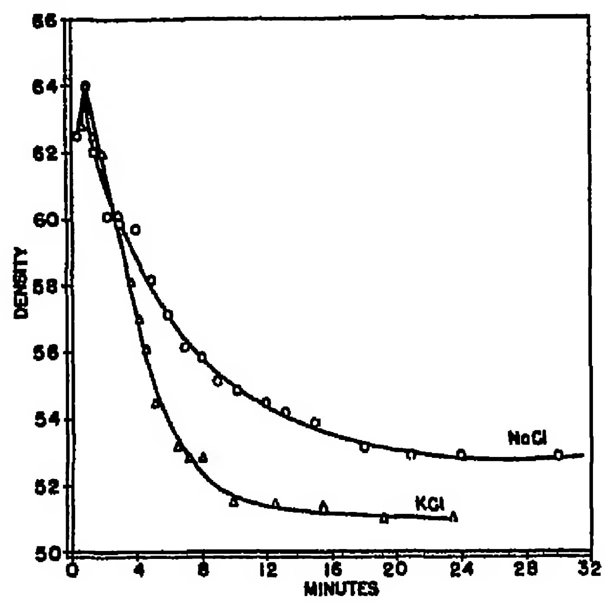

FIGURE 1. Hemolysis of Chelydra erythrocytes in 0.125 molal NaCl and 0.126 molal KCl. 29° C. pH 7.41. Solutions buffered with corresponding isosmotic phosphate in the proportion 9:1. The initial rise in the curves indicates preliminary shrinkage probably due to slight hypertonicity. The final values for optical density represent complete hemolysis for both solutions, the difference apparently resulting from difference in opacity of the ghosts.

The rate of hemolysis in any given electrolyte solution was always greater in an alkaline than in an acid solution. This could be shown by adjusting the pH with HCl or the hydroxide of the cation being studied, or by use of phosphate buffers. Figure 3 illustrates the effect in the case of KCl buffered with isosmotic phosphate.

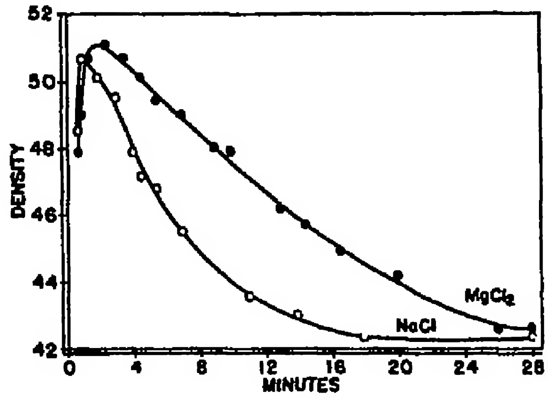

FIGURE 2. Hemolysis of Chelydra erythrocytes in 0.125 molal NaCl and 0.088 molal $MgCl_2$. 28° C. pH 7.41. Solutions buffered with 0.1 molal sodium phosphate 9:1.

NaCl behaved similarly. The rate showed little change within the range pH 7.8 to pH 6.7 but was definitely decreased at pH 5.9 to pH 6.1. The inhibitory effect of acidity was demonstrated in another manner (Fig. 4). Cells were placed in NaCl buffered to pH 7.0 with a trace of phosphate. After hemolysis was well under way acidified NaCl was added. Hemolysis was quickly arrested. On the addition of alkaline NaCl hemolysis was resumed at the original rate, indicating reversibility of the inhibition. If cells remain in the acid NaCl longer than about 10 minutes, hemolysis will be resumed at a very slow rate. When the pH is restored to the alkaline range after 50 minutes the former rapid rate is regained.

The effects of the cyanide and citrate of sodium and potassium were compared with the corresponding chloride at pH 7.25 or 7.4. Isosmotic mixtures of chloride and cyanide containing 0.019 molal cyanide exhibited a hemolytic action similar to that of isosmotic chloride. The same results were obtained with cells exposed to

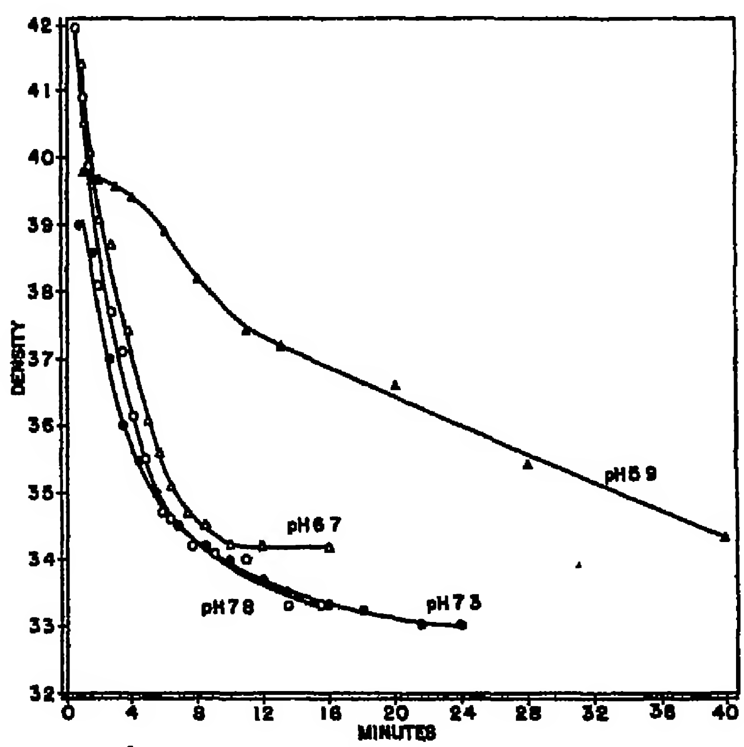

FIGURE 3. Hemolysis of Chelydra erythrocytes in 0.126 molal KCl buffered with 0.1 molal potassium phosphate 9:1. 28° C.

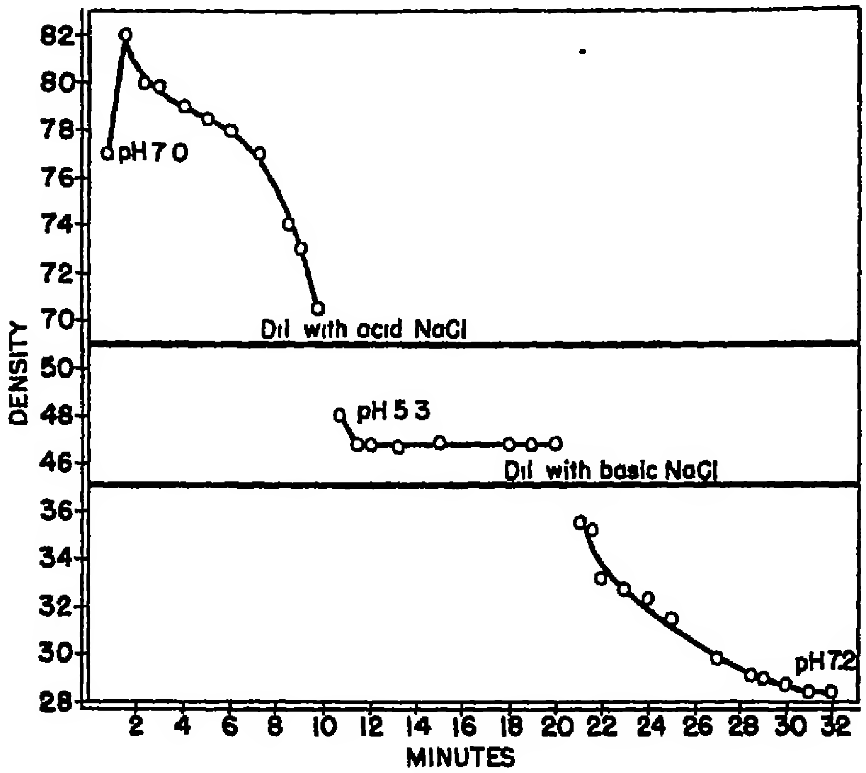

FIGURE 4. Effect of acidity on hemolysis of Chelydra erythrocytes in 0.125 molal NaCl. Ten minutes after the erythrocytes were mixed with 4.0 ml. NaCl solution (pH 7.0), and during the course of rapid hemolysis, 3.0 ml. of acid NaCl was added bringing the pH to 5.3. Hemolysis was arrested within one minute. Addition of 1.9 ml. of alkaline NaCl at 20 minutes raised the pH and hemolysis continued immediately. Dilution gave a marked change in optical density as indicated. The initial rise in the curve indicates shrinkage probably due to hypertonicity. 21° C.

the same concentration of cyanide in Ringer for 30 minutes before adding the hemolytic solution. The absence of any marked effect is not surprising in view of previous work with other electrolytes (Davson and Danielli, 1938; Hunter, 1947). Isosmotic sodium citrate gave slower hemolysis than NaCl, which may be the result of impermeability to the citrate ion as compared with chloride (Jacobs, 1940).

Sugars

When Chelydra erythrocytes are suspended in unbuffered isosmotic dextrose solution, agglutination occurs followed by hemolysis. The amount of hemolysis can be determined by removing the cells by centrifugation and measuring the concentration of hemoglobin in the solution. If the suspension is pipetted up and down, the masses of cells tend to break up and hemolysis is further increased. In dextrose buffered at pH 7.5 with a small amount of sodium phosphate, agglutination was no longer apparent; and, as might be expected from the effect of pH on hemolysis in salt solutions, hemolysis was more pronounced.[3] A comparison of the hemolytic action of various sugars, including one disaccharide, two hexoses, and two pentoses (Table I), showed that the pentoses differed markedly from the other sugars, hemolysis being absent in arabinose and very slight in xylose. Dextrose gave complete hemolysis. On centrifugation of such hemolyzing suspensions, a

[3] The buffer may well have an effect as an electrolyte.

jelly-like mass containing nuclei, many distorted and a few normal erythrocytes, was found. This contrasts with hemolysis in electrolytes in which normal ghosts were present, with about 50 per cent of the ghosts sphering in isosmotic $MgCl_2$.

Observations on agglutination indicate that differences exist between sugars in this respect as well as in their hemolytic action. Cells (0.07 – 0.08 ml.) suspended in Ringer were centrifuged briefly; the supernate was carefully removed and 8.5 ml. of isosmotic sugar solution was added. After stirring to give uniform cell distribution, the suspension was left undisturbed.[3] Results were similar for three individuals (Table I). While strict comparisons between certain sugars cannot be made because of differences in pH, it is apparent that all sugars cause agglutination, but all are not equivalent. Moreover, the effects of agglutination do not parallel hemolytic actions. So, for example, sucrose is hemolytic and weakly agglutinating; arabinose, on the other hand, has a greater agglutinating action without producing hemolysis.

TABLE I

Hemolysis and agglutination in isosmotic sugar solutions

Cells were removed from solutions after 3 hours and the optical density of the supernate was measured at 576 millimicrons.* pH 7.5-25° C. Blood from three turtles.

	Hemolysis		Agglutination	
	Sample 1	Sample 2		pH
dextrose	0.630	0.510	++++	5.4
sucrose	0.344	0.378	+	5.7
d-galactose	0.325	0.163	+	4.5
d-xylose	0.087	0.017	++++	4.4
l-arabinose	0.007	0.005	+++	5.7
Ringer	0.017	0.014	0	5.7

* After initial mixing the suspensions were left undisturbed to minimize hemolysis and then centrifuged. Centrifugation may increase the hemolysis slightly. (Collier, 1948. Personal communication.) The absorption spectrum was determined for one sample of blood, and maxima were located at 543 and 576 millimicrons. These agree closely with values given for human blood.

The influence of electrolytes on agglutination may be shown by adding one part of isosmotic NaCl, KCl, $MgCl_2$ or $CaCl_2$ to 14 parts of isosmotic dextrose. Agglutination was inhibited somewhat by the addition of electrolytes in all cases and was less pronounced in the presence of NaCl and $MgCl_2$ than with the other salts (Fig. 5). But in no instance was agglutination completely prevented. Microscopic observations indicate similar differences (Figs. 6 and 7). Quantitative aspects of this effect, previously studied on other cells (Radsma, 1918), have not been investigated.

[3] Sedimentation rate fails to give a measure of the extent of agglutination in this blood inasmuch as some cells do not agglutinate and therefore sediment relatively slowly, whereas the agglutinated masses in the same suspension fall rapidly or adhere to the wall of the tube. Adherence to the wall is observed in vertical tubes of 1.0 cm. or more in diameter.

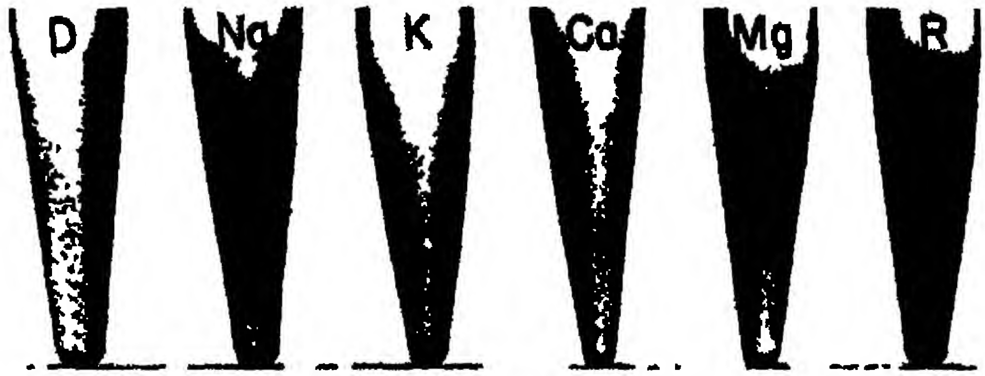

FIGURE 5.

FIGURE 6.

FIGURE 7.

FIGURE 5. Effect of salts on agglutination of washed Chelydra erythrocytes in dextrose solution. Isosmotic salt solutions were added to unbuffered dextrose (0.23 molal) in the proportion 1:14. D—dextrose; Na—NaCl + dextrose; K—KCl + dextrose; Ca—$CaCl_2$ + dextrose; Mg—$MgCl_2$ + dextrose; R—Ringer (unbuffered). After initial mixing tubes were left undisturbed. 26.5° C.

FIGURE 6. Chelydra erythrocytes in 0.23 molal unbuffered dextrose. Washed cells were mixed with dextrose solution and placed immediately on a slide without coverslip. Arrows indicate ghosts.

FIGURE 7. Chelydra erythrocytes in 0.23 molal unbuffered dextrose with isosmotic NaCl 14:1. Shrinkage may be noted.

DISCUSSION

Consideration of the mode of action of electrolytes and non-electrolytes on Chelydra erythrocytes is complicated by the fact that related substances may be strikingly different in their actions. Thus, no single scheme will serve to explain completely the action of sugars, nor can cation effects be interpreted adequately in

terms of valence. The latter is in contrast to human erythrocytes, in which ions of the same valence are alike in preventing loss of salts from cells in sucrose solution (Wilbrandt, 1940).

The effect of calcium in maintaining the normal permeability characteristics of the cell may be considered in relation to (1) the thickness of the ionic double layer and the adhesion of membrane components, and (2) crossbinding of anions within the membrane (Danielli, 1937, 1943). In both respects calcium has a more pronounced action than sodium or potassium. Following the Gibbs-Donnan equilibrium, with a change of medium from Ringer to isosmotic NaCl or KCl as was done in the present experiments, there will be a replacement of calcium in the surface layer. Compactness of the membrane will be decreased, which may in turn lead to an increase in permeability resulting in swelling and hemolysis. Such an increase in volume preceding hemolysis in these solutions may be readily observed under the microscope. Additional assumptions will be necessary to explain such differences as those found between the effects of calcium and magnesium on hemolysis and on sphering of ghost cells.

With sugar solution as the medium, the salt concentration essentially zero, and a constant anion concentration in the cell surface, there is to be expected from the Gibbs-Donnan equilibrium a decrease in membrane concentration of mono- and divalent metal ions, and an increase in surface acidity, which has been thought to be of sufficient magnitude to alter the proteins of the membrane and accordingly cell permeability (Danielli, 1937; Wilbrandt, 1940). However, in this cell an increase in acidity of the medium stabilizes the membrane, as shown by the acid inhibition of hemolysis in both electrolytes and non-electrolytes. At the same time the loss of metal ions would result in increased repulsive forces within the membrane, giving greater distances between molecules and an increase in permeability (Danielli, 1943). Even though it is assumed that the net effect favors an increased permeability, the present results are not completely explained inasmuch as all sugars should behave similarly, whereas some have been shown to be hemolytic while others are not.

Other factors to be considered are a differential permeability to sugars and effects of individual sugars on membrane structure. Any differences in permeability which may exist must involve factors other than molecular volume since this does not correlate with hemolytic action. So, for example, sugars which were least hemolytic (pentoses) have the smallest molecular volume. (See also Ulrich, 1934.) The agglutination of cells indicates an alteration of the cell surface by the sugar, the degree to which this occurs depending upon the particular sugar and ionic composition of the medium. Further, hemolysis in sugar, contrary to the results in electrolyte solutions, was characterized by disintegration of many of the cells, again pointing to a direct action on membrane structure.

The effect of acidity in decreasing hemolysis obtained with electrolyte solutions suggests that molecular rearrangements within the membrane may in part compensate for the lack of calcium. The fact that the erythrocyte is stable in certain Ca-free non-electrolytes indicates that it is not the absence of calcium *per se* which causes hemolysis in electrolytes, but rather the effect of other cations which may replace the calcium of the cell membrane and so increase its permeability. The cell, then, would be sensitive to lack of calcium only because of the ready replacement of its calcium by other cations.

Summary

1. The comparative hemolytic rates of *Chelydra serpentina* erythrocytes in isosmotic salt solutions as measured photometrically were KCl > NaCl > $MgCl_2$, and NaCl > Na_3 citrate. Hemolysis in cyanide (0.019 molal) was similar to that in chloride. No hemolysis occurred in isosmotic $CaCl_2$ and the addition of $CaCl_2$ to cells hemolyzing in Ca-free electrolyte solutions arrested hemolysis at once.

2. Hemolysis in sodium and potassium solutions was greatly retarded at about pH 6 and below.

3. The hemolytic potency of isosmotic sugar solutions (pH 7.4) was found to be: dextrose > sucrose > d-galactose > d-xylose with complete hemolysis in dextrose and none in l-arabinose in three hours.

4. Sugar hemolysis was accompanied by abnormal shape changes and disintegration of cells, whereas in Ca-free electrolyte solutions "normal" ghosts were found.

5. Agglutination occurred in unbuffered isosmotic sugar solutions, the extent depending upon the particular sugar. Agglutinating action was not correlated with hemolytic potency.

6. Results of experiments on the hemolytic and agglutinating properties of sugars indicate that certain sugars are not inert but have a definite action on the cell surface.

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EXPERIMENTS ON THE DETERMINATION AND DIFFERENTIATION OF SEX IN THE BOPYRID STEGOPHRYXUS HYPTIUS THOMPSON [1]

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One of the great controversies related to the Bopyridae (a family of isopod crustaceans, suborder Epicaridea), and one that has broader biological implications as well, is the question of sex-determination. Is sex already determined in the larval stage, or does the fate of the larva (i.e. whether it turns into a female or a male) depend on environmental influences? Can such external factors as the position the larva occupies or the nourishment it receives bring about sex reversal in an already sex-determined larva?

The chief views expressed in the past regarding this question are as follows:

1. Giard and Bonnier (1887) maintained that in the Bopyridae all free-swimming larvae are males. The first larva, however, that invades a particular host undergoes sex reversal and transforms into a large female without ever having functioned as a male. The next to come settles on this female and metamorphoses into an adult dwarf male which fertilizes the former.

2. Smith (1909) and Goldschmidt (1920) stated that all the species of Epicaridea, Bopyrina as well as Cryptoniscina, are protandric hermaphrodites, each individual being male while in a larval state, and then losing its male organization and becoming female as the parasitic habit is assumed. The females, therefore, result from males that have already functioned as males.

3. Hiraiwa (1936) believed that the free-swimming larvae are not males but are sexually undifferentiated, although the sex is already predetermined. Differentiation follows fixation, but is probably not due to environmental factors.

4. Recently, Caullery (1941), impressed by the influence of association on sexuality as exhibited in such animals as Bonellia, Crepidula, and Ophyotrocha, made the suggestion that the sexes may not be fixed from the start, but that direct parasitism of a larva on a host entails differentiation into a female, and indirect parasitism, through the intermediary of a female on which it is stationed, entails differentiation into a male. Lacking direct evidence, however, he suggested an experimental approach to test the validity of this theory. He advised collecting the newly-arrived cryptoniscid larvae that can frequently be found in the brood pouch of a female bopyrid—larvae which according to this view would evolve into typical males under the influence of the environment—and placing them in contact with young crabs not yet parasitized. Caullery thought it probable that these larvae would fix to the crab and become females.

These suggestions of Caullery moved the writer to undertake a series of experiments with the larvae of *Stegophryxus hyptius* Thompson, an ectoparasite of the

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hermit crab *Pagurus longicarpus* Say. This work was carried on in the summer of 1946 at the Marine Biological Laboratory, Woods Hole, Mass. In addition to the experiment suggested by Caullery, reciprocal experiments were undertaken in which presumptive female larvae were taken from the host crab and transferred to the brood pouch of a female bopyrid to test the suspected masculinizing influence of the female on cryptonisci that attach to her body.

The present paper reporting on this work was ready for publication when the writer's attention was drawn to an article in Italian by Reverberi and Pitotti, which, although it appeared in 1942, had not been mentioned in the abstracting journals until 1947. This paper provides the first experimental verification of any of the proposed sex-determination theories with reference to the Bopyridae. The authors, working with *Ione thoracica* Montagu, showed that the control of sex-determination is environmental rather than genetic. However, since there are several points of difference between the biological cycles and sex phenomena of Ione and Stegophryxus, it was decided not to alter the present paper as originally written, but in the discussion and footnotes to draw a comparison between the results reported by Reverberi and Pitotti and our own.

Life Cycle of Stegophryxus

Only about 1.5 per cent of *Pagurus longicarpus* at Woods Hole are parasitized by *Stegophryxus hyptius*. Thompson (1901), in his original description of the species, gave an account of the morphology of the adult female, adult male and some of the immature forms, but the life cycle has heretofore not been discussed.

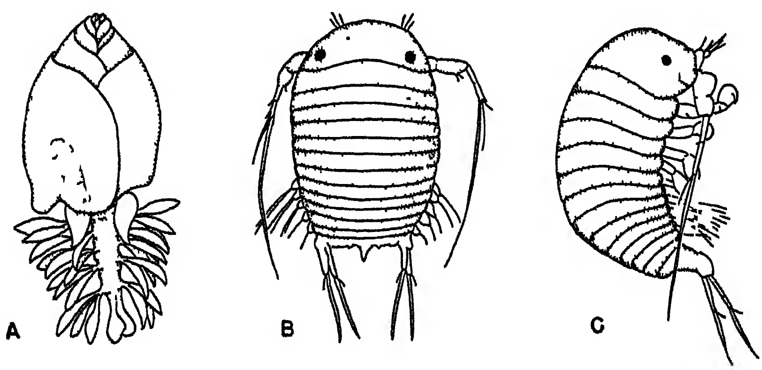

FIGURE 1. Adult female and epicaridium larva of *Stegophryxus hyptius*.
A. Ventral view of adult female. The dwarf male, although not visible externally, is shown within the brood pouch by a dotted outline to indicate its position and relative size. × 5. B. The epicaridium or first larval stage shown in dorsal view. × 120. C. Lateral view of epicaridium larva. × 120.

The female Stegophryxus, as is the case in all bopyrids, is much larger than the male (Fig. 1A). It occurs on the abdomen of the hermit crab, to which it is attached, back downward, by its mandibles and legs. Its thorax is concealed ventrally by an enormous brood pouch, made up of five pairs of thin brood plates.

Within this brood pouch lies the slender dwarf male, whose function is not that of inseminating the female and then quitting her, but of remaining in readiness to fertilize the successive batches of eggs that are released into the brood pouch during the female's productive life. These eggs, within two weeks after fertilization, develop into first stage larvae that leave the mother and swim off. After an interval of about five days, the marsupium is again filled with eggs and another brood begins embryonic development.

The first larval stage is known as the *epicaridium*. It is a short, broad, semi-barrel-shaped larva (Fig. 1B) with sub-chelate pereiopods for clinging and with pleopods in the form of swimming organs. The epicaridium of Stegophryxus measures about 270 μ in length, 150 μ in breadth, and 120 μ in depth (not including the appendages). In this stage the young of Stegophryxus escape from the brood pouch and swim off as plankton organisms. In the laboratory, they quickly rise to the surface of the water and remain there floating or swimming about for days.

The subsequent history of the epicarid larva has not been investigated in Stegophryxus. It may attach to a pelagic copepod, undergo a molt, and become a *microniscus* larva, which, after feeding on the copepod, will eventually transform into a new larval stage known as the *cryptoniscus* that swims off to seek the definitive host. This type of development is known to occur in some of the Epicaridea (Sars, 1899; Caullery, 1907; Caroli, 1928; Reverberi and Pitotti, 1942). Or the epicarid larva may develop directly into a cryptoniscus larva, an abbreviated type of development which Hiraiwa (1936) believes is the case in most Bopyridae. We postulate the first alternative in the case of Stegophryxus because of the great difference in size between its epicaridium and cryptoniscus stages, a difference which can only be accounted for by assuming the existence of an intervening stage.

At any rate, however arrived at, the earliest larval stage of Stegophryxus that we find on the crab is the cryptoniscus. In this stage (Fig. 2) the parasite is typically isopod in its characteristics. It has an elongated body, dorsoventrally compressed, segmented and well chitinized. There are seven pairs of thoracic appendages (as compared with six pairs in the epicaridium) all similar in form, six pairs of uniramous natatory pleopods, and one pair of biramous uropods. The cryptoniscus measures about 680 μ in length, being therefore about two and one-half times longer than the epicaridium.

We have been able to distinguish three phases in the life of the cryptoniscus larva on the basis of color pattern which we shall designate as (1) the brown chromatophore phase, (2) the black chromatophore phase, and (3) the striped contracted phase.

The youngest cryptonisci, those that have recently settled on a crab, have a profusion of dark brown expanded chromatophores that cover the dorsal surface of the body in such a way as to leave an uncolored portion that resembles a cross (Fig. 2A). These chromatophores are present laterally on the head and segments 1, 4, 5, 6, 7, and 8; are present centrally as well as laterally on segments 12 and 13 and on the uropods; and are entirely absent from segments 2, 3, 9, 10, and 11. The general body color is pale yellowish and is due to another system of chromatophores, which are scattered over the integument without definite plan. The eyes are reddish brown.

In phase 2, the light colored cross-shaped pattern remains much as before, but most of the areas formerly occupied by brown chromatophores are now occupied

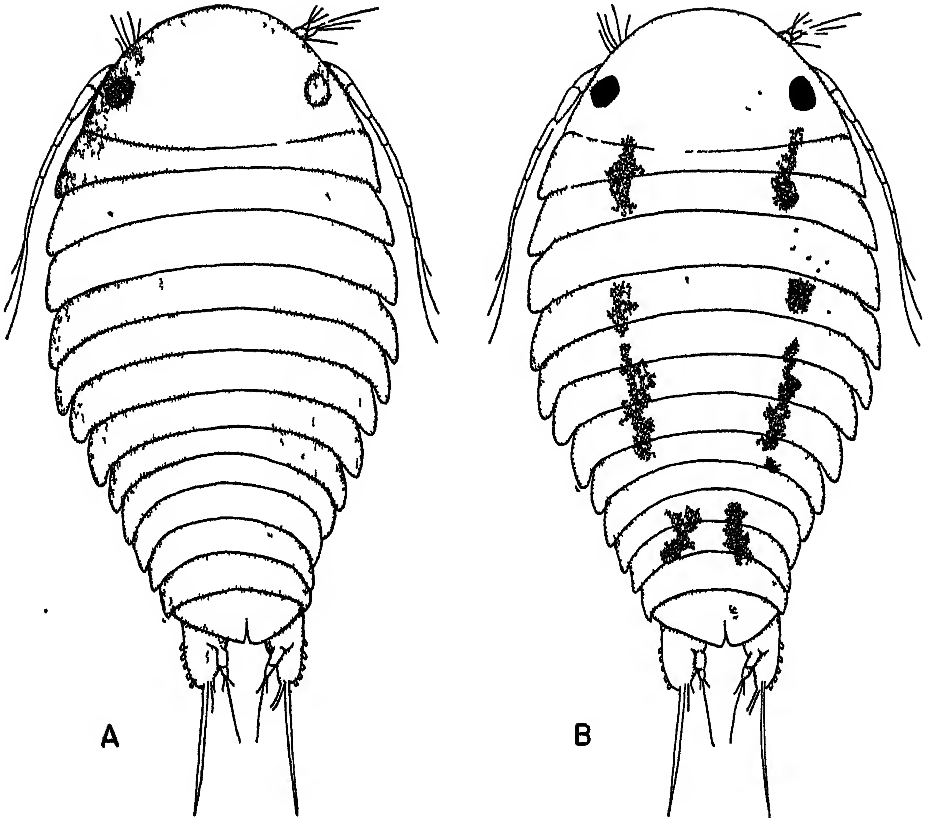

FIGURE 2. The cryptoniscus larva of *Stegophryxus hyptius*
A. Young larva showing color pattern when in phase 1 The light cross-shaped area is devoid of chromatophores. B. Older cryptoniscus in phase 3 with contracted melanophores forming an interrupted stripe on each side

by expanded black chromatophores. Their distribution is as follows: present laterally on the head and segments 1, 4, 5, 6, 7, and 8; present centrally on segments 11 and 12; absent from segments 2, 3, 9, 10, 13 and the uropods. The eyes have also become black. The yellow chromatophores are now more noticeable and have become restricted to segments 1 to 11 inclusive where they are present laterally.

In phase 3 (Fig. 2B) the black chromatophores are much fewer in number and are all in the contracted state. They form a broken chain on each side of the body about midway between the center and margin of the dorsal surface, reaching from segment 1 to segment 8 inclusive, but absent on segment 3. On segments 10 and 11 there are a few black chromatophores centrally located. Yellow chromatophores are intermingled with the black in the same chain but extend from segment 1 to segment 11. The eyes are black. In this stage the cryptoniscus is ready for the molt which will transform it into a juvenile female of the first postlarval stage.

No structural differences have been detected in these three cryptoniscid stages. Since neither the brown nor the black chromatophores lose their color in alcohol, they are no doubt melanophores which presumably differ only in the amount of melanin present

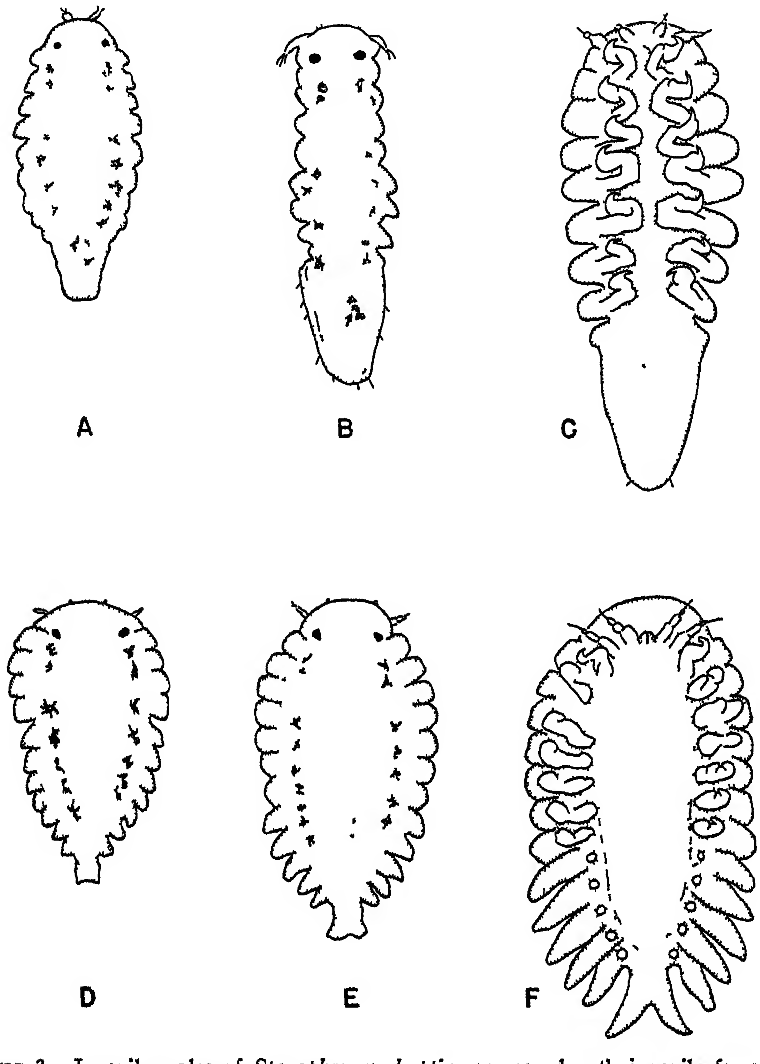

FIGURE 3 Juvenile males of *Stegophryxus hyptius* compared with juvenile females of the same species and same degree of development

A. Juvenile male in first post-cryptoniscid instar Specimen drawn measured 0 85 mm. Dorsal view. B Older juvenile male measuring 1 07 mm. in length. Dorsal view. C. More advanced juvenile male measuring 1.4 mm. in length Ventral view D Juvenile female in first post-cryptoniscid instar Specimen drawn measured 0 85 mm Dorsal view E. Older juvenile female, 1.01 mm. in length. Dorsal view. F. More advanced juvenile female measuring 1 3 mm. in length. Ventral view.

The juvenile female into which the cryptoniscus transforms is shown in Figure 3D. It is broader than the cryptoniscus and the pleon lacks uropods and possesses only stump-like rudiments of pleopods. The terminal segment is extended into a tail-like outgrowth ending in a shallow notch. The animal is whitish with color pattern differing little from that of the last stage cryptoniscus More advanced juvenile females are shown in Figures 3E and 3F.

About ten per cent of the crabs examined in the summer of 1946 were infested with one or more cryptonisci. This was fortunate, insofar as experimental possibilities were concerned, since infective cryptonisci have rarely been reported. Bonnier, for example, having studied about eighty species, came across cryptoniscus larvae that had recently attached to the host only twice. Hiraiwa never found them during five years' study of *Epipenaeon japonica*. The cryptonisci of Stegophryxus may be found on almost any part of the crab, but only those attached to the pleopods have actually settled down. The others are transients or new arrivals that wander off at the slightest disturbance. Even those on the pleopods, while more permanent than the others, are apt to leave when disturbed. The older the cryptoniscus, the more fixed in position it is, and if found attached to the last pleopod (third abdominal appendage of males, fourth of females), the favorite resting site, one can presume that it will remain there, barring accidents, until it eventually transforms into a female. Only after the juvenile female stage is assumed does the parasite leave the pleopod to fix itself permanently on the abdomen proper of the host.

Cryptonisci destined to become functional males are those found attached to a young or mature female. They are identical in form, size, and color pattern with those found on the crab. They too pass through the same three phases, but metamorphose into juvenile males. The first male instar is shown in Figure 3A. It is narrower in form than the corresponding female instar and has a strikingly different type of pleon which is tongue-shaped and rounded at the tip. More advanced juvenile males are shown in Figures 3B and 3C.

Transformation of Presumptive Females Into Males

The first question to be answered experimentally was whether or not the cryptonisci found on normal crabs, that is on crabs not infested with a female Stegophryxus, could be transformed into males. Such larvae in all likelihood would be presumptive females. If removed from the crab and transferred to the brood pouch of a female bopyrid, would these cryptonisci metamorphose into males?

I. In the first series of experiments, cryptonisci taken at random from normal crabs were placed in a dish with a crab parasitized by a mature Stegophryxus. The male was first removed from the brood pouch of the female to prevent interference. This was necessary because if the adult male is allowed to remain, although cryptonisci will attach as freely as when no male is present, they will enjoy only a relatively brief period of attachment before they are driven off.

Four experiments of this type were conducted. In all cases the greater proportion of the cryptonisci attached to the female bopyrid and the greater proportion likewise entered the juvenile male phase. But, after varying lengths of residence in the brood pouch and correspondingly varied degrees of attainment of the male phase, all but one out of each lot eventually deserted the female. The one that re-

mained in unchallenged possession eventually became a mature male, and in cases where the experiment was continued long enough this male functioned as such and successfully fertilized the eggs of its consort which then developed normally into epicaridium larvae.

It will be sufficient to cite one experiment of this series in detail. This experiment was begun July 13, 1946 with five cryptonisci taken at random from unparasitized crabs and placed in a dish with a crab having a mature Stegophryxus (male removed) whose brood pouch contained late embryos.

July 14. Two cryptonisci have attached to the female Stegophryxus.

July 15. Four cryptonisci now present on the bopyrid. Epicarids are hatching.

July 16. Three cryptonisci remain within the now empty brood pouch. They have developed to the black pigment stage.

July 17. Metamorphosis of cryptonisci continuing; one, at least, has molted.

July 19. The three cryptonisci have entered the juvenile male phase and one is slightly more advanced than the others.

July 24. The three juvenile males are still present and continuing their development.

July 26. One of the juvenile males has disappeared. One of the two remaining ones is permanently removed for examination and drawings are made of it.

August 9. The brood pouch of the female bopyrid is now filled with eggs. (This means that the male has reached maturity.)

August 11. The male was removed for measuring and returned to the brood pouch. Its length is 2.28 mm.

August 24. Development of the eggs has continued normally and today the epicarid larvae are released.

August 26. The male now measures 2.37 mm. Experiment discontinued.

Similar results were obtained when female bopyrids, found in nature with a retinue of cryptonisci present in the brood pouch, were kept under observation. In one case a female Stegophryxus, non-ovigerous and lacking a male, had 18 cryptonisci attached to it. The daily count showed a reduction as follows: 18, 16, 14, 11, 9, 6, 4, 2, 2, 2, 2, 2, 2, 1. The remaining one reached maturity two weeks later and fertilized the eggs of the female which were not released until that time. Another reduction from an initial natural retinue of eight cryptonisci occurred as follows: from 8 to 2 in four days, but these two persisted for 11 more days to become juvenile males, then one disappeared. The survivor became a mature male.

These experiments and observations, while they shed some light on the problem at hand, are inconclusive evidence for or against any theory of the sexual nature of the cryptonisci. They show that cryptonisci that enter the brood pouch of a female metamorphose in the male direction, but what of those that leave early or fail to enter? Could not they be predetermined females unresponsive to masculinizing influences?

II. To settle this point, it was decided to experiment with single cryptonisci. Moreover, only cryptonisci found clinging to the posterior pleopod of a normal crab were used. Nine experiments were undertaken. In five of these the cryptoniscus selected for insertion in the brood pouch of a female was in the brown chromatophore stage; the four other cryptonisci were in the more advanced black stage.

Each of the five Stage 1 cryptonisci remained in the brood pouch and made no efforts to crawl out. One was removed after six days, one after eight, one after eleven, and two after twelve days. Each one had metamorphosed into a male, whose size and extent of development was proportional to the length of time spent in the brood pouch. Those that had been on the female for eleven or twelve days had reached a size of from 1.5 mm. to 1.8 mm.

The experiments with Stage 2 cryptonisci gave different results. In three cases the cryptoniscus crawled out of the brood pouch within a day or two and was either lost or found clinging to the crab instead. One experiment yielded positive results. This cryptoniscus refused repeatedly to attach to the female, but after each escape it was returned to the brood pouch. Finally it remained there, and, eventually, 28 days later, had become a 2 mm. male.

The positive results obtained with the five Stage 1 cryptonisci strongly indicate that cryptonisci that would ordinarily become females can readily be transformed into males through attachment to the body of the female bopyrid, provided the transfer is effected at an early age. Even the one success with a Stage 2 cryptoniscus confirms this. It must be concluded that after a certain period of parasitism on the crab the cryptoniscus becomes female-determined and the direction of its sex development can no longer be changed under ordinary conditions. Subsequent experiments, using juvenile females for transfer, instead of cryptonisci, support this view and will now be briefly recounted.

III. Four attempts were made to transform juvenile females into males. All were completely unsuccessful. Juvenile females in the early post-cryptoniscid phase were used, before they had developed far enough to leave the pleopod of the host for permanent attachment on the abdomen.

One female remained inside the brood pouch for one day, crawled to the exterior and remained there one day, then disappeared.

One left the brood pouch the day after transfer and attached to the abdomen of the crab where it remained for eight days, when the crab died.

One left the brood pouch on the second day and attached to the underside of the telson of the crab. It remained there until the experiment was discontinued 12 days later, and grew from an initial size of .85 mm. to 1.4 mm.

The fourth one was transferred to the brood pouch together with the pleopod to which it was attached. This female remained for five days, then disappeared and could not be recovered for examination.

It would seem that juvenile females are averse to becoming ectoparasites of other more mature females. They leave such an unnatural situation to return to direct parasitism on the crab.[2] There is no evidence that any of the four were modified by their brief sojourn in the brood pouch of another female.

[2] Reverberi (1947) came to the same conclusion with regard to Ione. However, he then placed two females together *in vitro* apart from the host, one being a juvenile female and the other an adult from which the juvenile would have to derive its nourishment. As often as the adult died, another of the same age would be substituted. By this ingenious method he was able to maintain a direct association between a juvenile female and an adult Ione for several months. One case of definite sexual inversion resulted from many trials of this sort. This particular juvenile female underwent external changes and gradually took on the appearance of a male. When killed and sectioned after nearly four months under these experimental conditions, the individual was found to have normal testes partially filled with sperm.

Attempts to Transform Presumptive Males into Females

If presumptive female cryptonisci can be turned into males by altering the environment, the question naturally arises regarding the possibility of producing females from presumptive male cryptonisci. The method of experimentation would be to remove cryptonisci from the brood pouch of a female bopyrid and transfer them to a crab instead. This is the type of experiment on which Caullery pinned his hopes of verifying the theory of sex determination in Epicaridea based upon the type of association with the host.

When this was tried it invariably led to failure because the transferred cryptonisci did not remain attached to the crab long enough to show either positive or negative results. This failure to remain attached need not necessarily be attributed to aversion on the part of the cryptonisci for a strange environment. Indeed, the hazards in the case of direct parasitism on the crab are great. When it is recalled that approximately 10 per cent of the normal crabs have cryptonisci on their surface and only 1.5 per cent of all crabs are infested with female Stegophryxi, it becomes clear that many potential parasites are eliminated through environmental difficulties. Moreover, no success was achieved in numerous attempts to rear to the juvenile female stage cryptonisci found naturally attached to crabs. When crabs bearing cryptonisci are isolated in a dish and examined after a day or two, one finds that the cryptonisci have disappeared. Apparently they are eaten by the crab, since cryptonisci kept in dishes without crabs will remain alive for as long as two weeks. It may be remarked in passing that although isolated cryptonisci survive, they do not develop, nor pass from the brown to the black phase. It is significant that the juvenile female Stegophryxi naturally occurring on crabs can be reared without difficulty in the laboratory. They are actually fixed to the crab and have lost their ability to swim, whereas the cryptonisci, as explained before, are still active and only perch on the crab without fastening themselves to it. Should they disengage themselves even momentarily, they are in danger of being caught up by the currents passing through the gill chambers of the crab and swept in the direction of the crab's mouth. The mouth parts of the crab are in constant motion and any particle that comes in contact with them is trapped and masticated.

The failure of these experiments with cryptonisci removed from the brood pouch of a female and transfered to a crab may, therefore, with considerable assurance, be laid to experimental difficulties.[3] When the proper technique is worked out for Stegophryxus, which will eliminate the hazards facing cryptonisci that attach to crabs, we feel confident that presumptive male cryptonisci can be transformed into females.

[3] This is especially reasonable in view of the fact that Reverberi and Pitotti (1942) experienced a similar lack of success when they tried to implant the cryptoniscus larvae of Ione on the gills of Callianassa. The cryptonisci invariably failed to remain on the host (pp. 148–149). But when they used post-cryptoniscid stages they were successful in bringing about the transformation of juvenile males into females. Ione, unlike Stegophryxus, is a branchial parasite. When juvenile males of Ione, removed from adult females, were placed in the branchial cavity of the host, they soon attached to the branchiae, began to feed, and in the majority of cases remained there more or less permanently. Such males gradually became females.

The Fate of Supernumerary Males and Females

The first series of experiments reported above furnish evidence that although any number of cryptonisci may attach to the same female and develop into juvenile males, only one male is allowed to reach maturity. This point has not been realized by most previous investigators except Caullery (1941) and Reverberi and Pitotti (1942), and hence, in the older literature, several cases of supernumerary males associated with one female bopyrid are mentioned, with the inference that they are highly unusual or evidence of polyandry. Pérez (1924), for example, reports three instances of this from his own observations involving *Pleurocrypta porcellanae* Hesse, *P. galatheae* Hesse, and *Athelges lorifera* Hesse. In the light of recent work, these must be interpreted as cases of extra males in process of development before they have realized the full adult state, since all cases, when analyzed, resolve themselves into the stegophryxoid pattern, namely, one adult functional male accompanied by one or more smaller juvenile males or cryptonisci.

It must be concluded, therefore, that it is a general rule in the bopyrids that only one functional male is permitted at a time. Other potential males, temporarily tolerated, are expelled sooner or later. Whether the legitimate male, by virtue of its larger size, actually drives off the others, or whether they are repelled in some more refined manner is a question still to be answered.

Our observations on the question of excess females show that they, like the supernumerary males, are eliminated sooner or later, usually as juveniles Only one adult female is ever found on an individual host.

One hermit crab, collected August 23, 1946, carried a large ovigerous Stegophryxus and had in addition four juvenile females in various stages of development attached to the abdomen and pleopods. This crab was isolated for daily observations and in less than a week's time the four juvenile females had disappeared. Two other cases of parasitized crabs, each with a juvenile female present in addition to the adult female Stegophryxus, were observed under laboratory conditions. One juvenile persisted from July 24 to August 10 and grew considerably in size before it was eliminated; the other lasted from July 29 to August 11.

It is significant that the lost juvenile females could not be found in the dish in which the crab had been isolated. Perhaps they drop off and are eaten by the crab. It is more probable, however, that they are driven off by the mature male. One finds, on occasion, the mature male wandering about on the outside of the female brood pouch, and it is not unlikely that the male engages in occasional forays over the abdomen of the crab and drives off or destroys the excess females before they reach maturity.

Histological Observations [4]

Four cryptonisci and ten males, the latter selected to form a graded series of sizes ranging from 1.2 mm. to 2.7 mm., were sectioned and studied histologically to determine the sexual nature of the larvae and the organogenesis of the male gonads.

[4] The author gratefully acknowledges the assistance of Cornelius Sharbaugh, T.O.R., who, under our direction, prepared the slides and made the morphological studies on which this portion of the paper is based.

It was found that males of 2 mm.-length and over could be termed "adult" as judged by the length and development of the testes and the presence of spermatozoa in the vasa deferentia. In such males the reproductive organs are seen as a pair of long tubular masses, beginning in the first thoracic segment, and extending back into the seventh thoracic segment. The testes lie dorso-laterally adjoining the two liver tubes, one on each side of the animal, except for the anterior extremity of each, which occupies a ventro-lateral position in relation to the liver. In the sixth and seventh thoracic segments, the testes become vasa deferentia which open separately to the outside on the ventral surface of the seventh thoracic segment. The beginning of the vas deferens is often dilated to act as a temporary seminal vesicle.

The cells that make up the testis in the anterior-most portion of the organ are all of one type and equally distributed throughout the cross section. Elsewhere, spermatocytes, spermatids, and spermatozoa may be seen arranged in three distinct zones: spermatocytes in the inner zone next to the liver, spermatids in the middle, and spermatozoa in the outer zone (Fig. 4). The cells of the inner and middle zones are grouped into areas or patches, but those of the outer zone extend without interruption the entire length of the testis.

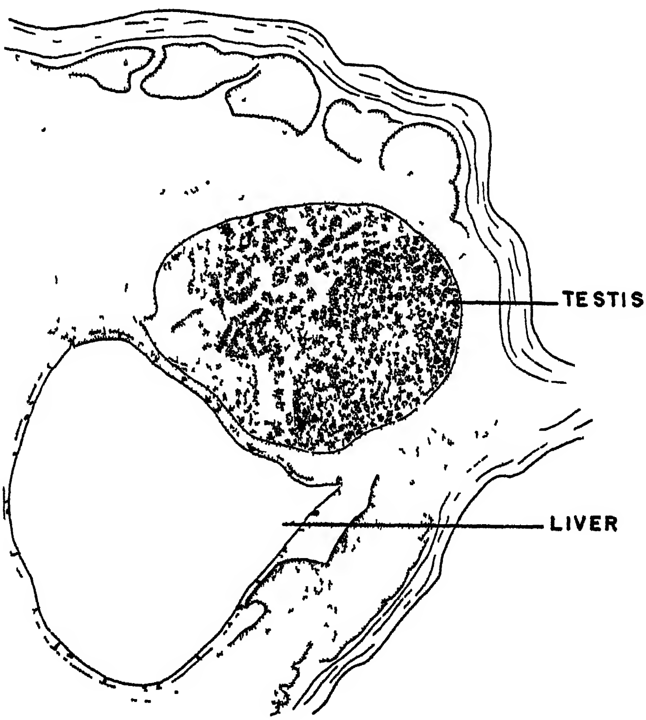

FIGURE 4. Right half of transverse section through the third thoracic segment of a male *Stegophryxus hyptius* measuring 2.7 mm. in length. The testis, dorso-lateral to the liver, shows the characteristic zonal arrangement: spermatocytes in the inner zone closest to the liver, spermatids intermediate in position, and spermatozoa in the outer zone. × 400.

Males of approximately 1.5 mm.-length are essentially similar to the larger males except that the testes are shorter, beginning in the second or third thoracic segment, and the three characteristic zones appear only in the posterior portion. Anteriorly, the testes contain spermatocytes and spermatids, but no spermatozoa.

In the smallest male examined, length 1.2 mm., the testis on the left side was undeveloped; that on the right was short and contained spermatocytes and spermatids only. These occupied a relatively short middle section, with anterior and posterior extremities appearing empty.

None of the four cryptonisci examined, three in cross section and one in longitudinal section, showed either gonads or groups of cells that might be regarded as traces of gonads.

Discussion and Conclusions

The experiments reported on here seem to indicate that the cryptoniscus larvae of Stegophryxus can develop in either of two directions: into males under conditions of parasitism on a female bopyrid, or into females, under conditions of parasitism on a hermit crab. As in the case of Bonellia (Baltzer, 1914), the larvae are apparently indifferent, with both sex potencies. Which potency will be realized depends on the conditions of the environment.

It is evident that the female bopyrid exercises a masculinizing influence on the cryptonisci directly attached to it. This influence does not extend to cryptonisci which are merely in the neighborhood of the female. They receive no male stimulus. To be affected, the cryptoniscus must be in actual contact with the female and perhaps even imbibe her body fluids. Certainly they and the juvenile males receive nourishment from the female, or how else could they grow to increase as much as threefold in size?

Whether nutritive conditions alone provide the masculinizing stimulus, or whether the controlling influence is a specific substance of hormonal nature, is a question requiring further experimental study. Nourishment, as pointed out by Zimmer (1927), is probably the determining factor in the production of females, but for the production of males it seems necessary to assume, as in Bonellia, the transfer of an actual secretion from the body of the female to the larvae that are attached to her, which acts as a specific masculinizing substance.

The sex-determination theory proposed by Giard and Bonnier (1887), namely, that the first larva that invades a particular host transforms into a large female, while the next to come settles on this female and metamorphoses into a dwarf male, is an explanation entirely too simple; but the first assumption, at least, is supported by the results of our experiments. The fate of subsequent comers is less certain. Conceivably, a second cryptoniscus might arrive shortly after the first and also settle down to become an incipient female. There must obviously be a time interval of some days before the first-comer has metamorphosed sufficiently to invite the attention of new arrivals. Let us say, therefore, that the fate of subsequent comers is in no way different from the fate of any cryptoniscus; viz. those that settle directly on a crab become female-determined, while those that settle on a female of their species become male-determined.

One of the objections to the theory of Giard and Bonnier has been the fact that two females are sometimes found on the same host. Thus Hiraiwa (1936) says:

"If the female in the (branchial) cavity makes the later invader into male, why are two females found in one and the same cavity?"

The answer to this objection is now clear. A female does not influence the sex of later invaders unless they settle directly on her body. Should a cryptoniscus settle on the crab, no matter how close in position to a large female, the cryptoniscus will not be affected by this proximity so far as its sex-determination is concerned.

The sex determination theory of Smith (1909) and Goldschmidt (1920) with reference to the Bopyridae requires no discussion. It is eliminated by the facts presented in describing the life cycle of Stegophryxus and has previously been sufficiently criticized by Hiraiwa (1936).

Hiraiwa's own theory, disclaiming as it does differentiation due to environmental factors, is not in harmony with the results of the experiments reported here. His assumption, however, that the free-swimming larvae are not males but are sexually undifferentiated is in agreement with our findings.

Caullery's theory (1941) finds ample confirmation in the results of our experiments. Although the exact experimental verification he hoped for has not yet been realized by us, the converse experiment of transferring cryptonisci from the host to the female bopyrid has yielded satisfactory evidence that the sexes are not fixed from the start.

Coming now to the studies on *Ione thoracica* made by Reverberi and Pitotti (1942) and Reverberi (1947), and their relation to the observations and deductions reported here on *Stegophryxus hyptius,* we find when we tabulate the two for comparison (Table 1) that the same general pattern runs through both. Some, perhaps most, of the differences that do exist are modifications to be expected when comparing species of different genera that differ also in habits and habitat. Thus, since Ione is a branchial parasite, the larvae have the opportunity of settling on the gills of the host (to become female-determined), on the female parasite itself (to become male-determined), or on the skin of the host (likewise to become male-determined). Stegophryxus presents a simpler condition since the female is attached not to the branchiostegite but to the abdomen of the host. Therefore only two substrates are selected for attachment by the larvae: the abdomen of the host or the female herself. If abundant nourishment is the factor that determines females and less abundant nourishment, as Reverberi and Pitotti at first thought, determines males, it is hard to understand why the abdomen of Pagurus should furnish abundant nourishment to the cryptonisci of Stegophryxus while the abdomen of Callianassa should not likewise yield abundant nourishment to cryptonisci of Ione. Later (1947), Reverberi, as a result of further experiments, came to the conclusion that the larvae that attach to the female parasite are masculinized by a sex-determining substance produced by the female rather than by "less abundant nourishment"; but the problem of the so-called "complementary males" on the skin of Callianassa is still very puzzling.

Reverberi's experiments on Ione were made almost exclusively with the older post-cryptoniscid larvae; ours dealt almost entirely with earlier larvae in the cryptoniscus stage. Since the larvae on the body of Callianassa were already presumptive males, his chief experiments were to make females out of them. In Stegophryxus, on the other hand, the cryptonisci on the body of the host are presumptive females, hence our main experiments were to make males out of them. All in all, the two studies complement and illuminate each other. Together they

TABLE 1

A comparison between Ione thoracica and Stegophryxus hyptius with respect to sex-determination. Data for Ione compiled from the papers of Reverberi and Pitotti (1942) and Reverberi (1947)

Ione thoracica	*Stegophryxus hyptius*
1. Adult female lives in the branchial cavity of Callianassa.	1. Adult female lives on the abdomen of Pagurus.
2. Females result from cryptonisci that settle on the gill of the host.	2 Females result from cryptonisci that settle on the abdomen of the host.
3. Females secrete a diffusible substance capable of attracting free cryptoniscus larvae.	3. Same.
4. Cryptonisci that attach to the female become males.	4. Same.
5. Only one adult male is retained.	5. Same.
6. Cryptonisci that attach to the skin of the host became complementary males, capable of replacing the lost male of an adult pair.	6. Cryptonisci that attach to the skin of the host become juvenile females. Replacement of lost males occurs from free cryptonisci only.
7. Juvenile males, removed from adult females, can be transformed into females by placing them on the gills of the host.	7. No evidence that juvenile males can undergo sex reversal, but presumptive male cryptonisci could probably be transformed into females if they left the female and obtained nourishment from the host instead.
8. A widowed male can become female if it succeeds in attaching to the host gill and getting abundant nourishment.	8. It is unlikely that males, once differentiated, could undergo sex reversal.
9. The few comparable experiments reported did not yield results.	9. Presumptive female cryptonisci become males when removed from the host and placed on a female parasite.
10. Evidence supplied in 1947 that differentiated females can undergo sex reversal.	10. No evidence that differentiated females have the capacity for reversal to male.
11. Larvae that engorge host blood directly become females if nourishment is abundant (taken from gills), but become complementary males if nourishment is less abundant (taken from skin of host).	11. All larvae in a position to feed on the host directly become females. Only larvae that attach to the female become males.
12. Reverberi first attributed maleness, when larvae are attached to a female, to "less abundant food," but later (1947) explained it as due to masculinizing substance.	12. Data favors hypothesis of a masculinizing substance produced by the female.
13. Gonad tissue first appears in older post-cryptoniscid forms and the earliest gonad is indifferent.	13. Cryptonisci lack gonads. The earliest gonad in juvenile males is a testis. No data presented on the earliest type of gonad in a juvenile female.
14. Sex-determination is environmental, comparable (with modifications) to that described for Ophryotrocha.	14. Sex-determination is environmental, comparable (with modifications) to that described for Bonellia.

fulfill the expectations of Caullery and reveal a fundamental plan of sex-determination and sex-differentiation, a plan, however, that can be expected to exhibit minor variations when utilized by different genera of Bopyridae.

SUMMARY

Stegophryxus hyptius Thompson, an ectoparasite on the abdomen of the hermit crab *Pagurus longicarpus* Say, seeks the definitive host as a cryptoniscus larva.

The free-swimming cryptonisci are sexually undifferentiated and sexually undetermined. Differentiation follows fixation and is dependent on environmental factors. These conclusions are justified on the basis of the following observations and experiments.

1. Cryptonisci that settle directly on the host develop into females; those that attach to a female bopyrid develop into males.

2. Changes in the color pattern of the cryptonisci following fixation furnish criteria for judging the length of time they have been subjected to a particular environment.

3. Histological examination of the cryptoniscus shows that the gonads are not yet present as recognizable structures.

4. Presumptive female cryptonisci, if removed from the host at an early age and transferred to the brood pouch of a female Stegophryxus, will transform into males.

5. The failure of the converse experiment involving transfer of presumptive male cryptonisci from the female parasite to the host can definitely be laid to experimental difficulties.

6. The factor that determines maleness is a masculinizing substance imbibed with food from the female, but this substance does not act at a distance.

7. Attachment of supernumerary females as well as excess differentiating males is terminated sooner or later so that a particular crab is host to only a single adult female paired with one functional male.

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STUDIES ON MARINE BRYOZOA, III. WOODS HOLE REGION BRYOZOA ASSOCIATED WITH ALGAE

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Introduction

Heretofore there has been no extensive study made of the association between bryozoa and algae, except for the studies by Joliet (1877) and Prenant and Teissier (1924, 1927, 1932), although incidental association notes are scattered throughout taxonomic papers.

The purposes of the present study are several: (1) to note any association that may exist between algae and certain bryozoa (bryozoa likely to be encountered in the Woods Hole region); (2) to aid collectors of bryozoa, since in some instances by collecting specified algae one is almost sure to find a number of desired bryozoa; (3) to make any observations possible on the tentacle number, the occurrence of embryos, larvae and ovicells in specimens collected during the summer months, and (4) to report any additional species from the collection area.

Collection Data

The materials used in this study were marine algae of three general groups: (1) freshly collected specimens; (2) dry, pressed specimens mounted at various times in the past on herbarium sheets; and (3) specimens preserved in the Botany Course Stock Collection of the Marine Biological Laboratory (M. B. L.) of Woods Hole, Mass. More species were examined than are here recorded but only those 37 algal species which had bryozoa growing on them are here listed.

Some of the fresh material was obtained in the intertidal zone by shore collecting; some had drifted in from deeper waters some distance from shore; and some had been dredged with a scallop dredge from waters about 20 to 60 feet in depth.

Some of the material was collected by the authors, some by the M. B. L. Invertebrate Zoology and Botany classes on their field trips, some by the M. B. L. Supply Department and Collecting Crew, and one algal species by Dr. Maxwell Doty of Northwestern University. To all these the authors wish to express their most sincere appreciation, and especially to Dr. William Randolph Taylor of the University of Michigan for very helpful suggestions, continued kindly interest, and for specimens of *Membranipora tuberculata* and their algal hosts from his own collection.

The *Laminaria longicruris* was brought in by Dr. Doty from Race Point, near Provincetown, Mass. on VIII–18–1947. The Rye Ledge, Rye, New Hampshire specimens of *Chondrus crispus, Phycodrys rubens* and *Phyllophora membranifolia* were collected by the junior author on IX–30–1945. The New Rochelle, New York specimens of *Ascophyllum nodosum, Ascophyllum Mackaii, Chondrus crispus* and *Laminaria Agardhii* were observed on IX–16 and 22–1945, and on X–3–1946 by the senior author. The remainder of the specimens were collected in the waters around and between North Falmouth, Martha's Vineyard, Woods Hole, Vineyard Sound, Buzzards Bay, Penikese Island and New Bedford, all in Massachusetts, during the summer months, between June 30 and August 31 approximately, over a period of several years (Sept. 1874, 1916, 1930, 1935, 1936, 1938, 1939, 1944 through 1947). Specimens from the five earliest years came from the M. B. L. Botanical Collection, some from wet mounts and some from dried herbarium mounts. Exact records, dates and collection numbers of all these algal specimens are on file, but only a very condensed amount of these data is included in Tables I to IV, to save space.

The senior author identified the bryozoa, the junior author classified the algae and collected many of them.

TABLE I

Collection sites and bryozoa associated with green algae

	Cladophora gracilis f. *tenuis*	*Enteromorpha intestinalis*	*Ulva Lactuca* var. *rigida*	Total No. green algae having this bryozoan species
Bowerbankia gracilis		x	x	2
Bugula turrita	x	x	x	3
Cryptosula pallasiana		x	x	2
Flustrella hispida			x	1
Pedicellina cernua	x			1
Total No. bryozoan spp. on this alga	2	3	4	
Collected at Woods Hole, Mass.	x	x	x	

To date there have been reported 84 species of marine bryozoa from the Woods Hole region, by Osburn (1912) mostly, and by Rogick (1945a, 1948). Some of the Woods Hole species have been previously reported from such widely separated regions as the coast of Africa, Australia, the Azores, Brazil, Denmark, Great Britain, Japan, New Zealand, Panama, Zanzibar, and the Pacific coast of North America. These were reported from various substrates as shells, rocks, hydroids, algae, various animals, piles, and other submerged objects. The present study was mainly directed toward finding the exact algal species on which bryozoa grow. Previous reports from the Woods Hole region occasionally did indicate the algal genera but rarely the species on which the bryozoa occurred. The following lists of algae and bryozoa are of species collected or examined for the present paper.

Below follows a list of 37 algae on which were commonly found various bryozoans. Taylor's (1937) classification is used.

TABLE II

Collection sites and bryozoa associated with brown algae

	Ascophyllum Mackaii	*Ascophyllum nodosum*	*Chorda Filum*	*Cladostephus verticillatus*	*Desmarestia aculeata*	*Fucus evanescens*	*Fucus vesiculosus*	*F. vesiculosus spiralis*	*Laminaria Agardhii*	*Laminaria longicruris*	*Sargassum Filipendula*	Total No. brown algal spp. associated with this bryozoan
Aetea sica		x	x	x			x	x	x		x	7
Aeverrillia armata									x			1
Aeverrillia setigera		x							x		x	3
Alcyonidium polyoum									x	x		2
Bowerbankia gracilis		x	x	x			x	x			x	6
Bowerbankia imbricata							x		x		x	3
Bugula cucullifera						x				x		2
Bugula flabellata							x					1
Bugula turrita		x	x				x	x	x		x	6
Callopora aurita									x			1
Cribrilina punctata									x			1
Crisia eburnea		x			x				x		x	4
Cryptosula pallasiana	x	x						x	x			4
Electra hastingsae								x	x			2
Electra pilosa		x			x	x		x	x	x	x	7
Flustrella hispida		x					x					2
Hippothoa hyalina		x						x	x		x	4
Membranipora lacroixii(?)	x											1
Microporella ciliata									x			1
Pedicellina cernua		x					x		x			3
Schizoporella biaperta					x				x			2
Schizoporella unicornis								x	x			2
Scruparia ambigua					x	x			x			3
Scruparia clavata									x			1
Smittina trispinosa									x			1
Total No. bryozoan spp. found on this alga	2	10	3	2	4	3	7	8	20	3	8	
Collected at Woods Hole		x	x	x			x	x	x		x	
Collected at North Falmouth		x						x			x	
Collected in Vineyard Sound			x		x	x					x	
Collected at Martha's Vineyard					x	x	x		x			
Collected at Penikese Island								x				
Collected at Provincetown										x		
Collected at New Rochelle	x	x							x			

TABLE III

Collection sites and bryozoa associated with red algae

	Agardhiella tenera	*Callithamnion roseum*	*Ceramium rubriforme*	*Cramium rubrum*	*Champia parvula*	*Chondrus crispus*	*Corallina officinalis*	*Cryptopleura* sp.	*Cystoclonium purpureum cirrhosum*	*Gracilaria confervoides*	*Gracilaria foliifera*	*Lithothamnium* sp.	*Phycodrys rubens*	*Phyllophora Brodiaei*	*Phyllophora membranifolia*	*Plumaria sericea*	*Polyides rotundus*	*Polysiphonia elongata*	*Polysiphonia nigra*	*Polysiphonia nigrescens*	*Polysiphonia varegata*	*Rhodomela subfusca*	*Rhodymenia palmata*	Total No. red algal spp. associated with this bryozoan
Aetea sica				x		x			x	x		x	x	x	x					x	x	x		11
Aeverrillia armata															x									1
Aeverrillia setigera					x	x									x					x		x		5
Alcyonidium polyoum						x								x	x									3
Bowerbankia gracilis	x			x	x	x	x		x		x			x	x					x				10
Bowerbankia imbricata						x					x													2
Bugula cucullifera															x								x	2
Bugula turrita						x			x				x	x	x		x			x				7
Callopora aurita													x		x									2
Cellepora dichotoma						x				x				x	x									4
Cribrilina annulata													x											1
Cribrilina punctata						x							x	x	x						x		x	6
Crisia eburnea				x	x	x	x		x	x			x	x	x	x			x	x	x		x	14
Cryptosula pallasiana						x			x					x	x						x			5
Electra pilosa						x			x				x	x	x	x			x		x	x	x	10
Flustrella hispida						x									x									2
Hippoporina contracta														x	x									2
Hippothoa hyalina				x		x	x		x	x			x	x	x		x	x			x	x	x	13
Lichenopora hispida													x		x									2
Membranipora lacroixii(?)						x									x									2

TABLE III—*Continued*

	Agardhiella tenera	*Callithamnion roseum*	*Ceramium rubriforme*	*Ceramium rubrum*	*Champia parvula*	*Chondrus crispus*	*Corallina officinalis*	*Cryptopleura* sp.	*Cystoclonium purpurcum cirrhosum*	*Gracilaria confervoides*	*Gracilaria foliifera*	*Lithothamnium* sp.	*Phycodrys rubens*	*Phyllophora Brodiaei*	*Phyllophora membranifolia*	*Plumaria sericea*	*Polyides rotundus*	*Polysiphonia elongata*	*Polysiphonia nigra*	*Polysiphonia nigrescens*	*Polysiphonia variegata*	*Rhodomela subfusca*	*Rhodymenia palmata*	Total No. red algal spp. associated with this bryozoan
Membranipora tuberculata								x																1
Microporella ciliata						x								x	x								x	4
Pedicellina cernua						x			x		x			x	x					x	x			7
Schizoporella biaperta						x	x		x				x	x	x					x				7
Schizoporella unicornis						x			x						x								x	4
Scruparia ambigua		x	x	x		x			x							x			x		x			8
Smittina trispinosa						x								x	x									3
Total No. bryozoan spp. on this alga	1	1	1	5	3	20	4	1	11	4	3	1	10	15	23	3	2	1	3	7	8	4	7	
Collected at Woods Hole	x			x	x	x				x	x	x		x	x					x	x	x		
Collected at Martha's Vineyard		x	x	x		x	x		x				x	x		x	x	x	x		x		x	
Collected in Vineyard Sound				x											x								x	
Collected at North Falmouth						x			x											x		x		
Collected on Penikese Island						x																		
Collected at New Bedford	x														x					x				
Collected at New Rochelle						x																		
Collected at Rye, N. H.						x							x		x									
From Puerto de la Paloma, Uruguay*								x																

* Specimens sent by Dr. W. R. Taylor.

TABLE IV

Collection sites and other Bryozoan data

	Penikese Island, Mass.	New Bedford, Mass.	North Falmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers. See under each bryozoan species discussion	Tentacle number. Personal observations unstarred, others starred	In filled-ovicell, embryo-producing or larva-releasing stage at Woods Hole
1. *Aetea sica*			x	x	x	x					18		9–11	July 30–Aug. 6±
2. *Aeverrillia armata*		x			x						2		8	
3. *Aeverrillia setigera*		x				x					8		8	
4. *Alcyonidium polyoum*		x				x	x		x		5	5	16, 20*	
5. *Bowerbankia gracilis*		x	x	x	x	x					18		8	
6. *Bowerbankia imbricata*					x	x	x				5	6	10	All of Aug.±
7. *Bugula cucullifera*		x		x					x		4		13	Aug. 1–18 ±
8. *Bugula flabellata*					x						1			June 1–Nov. 15
9. *Bugula turrita*			x	x	x	x					16		14	June 30–Aug. 15±
10. *Callopora aurita*					x	x		x			3			
11. *Cellepora dichotoma*					x	x					4			July 25±
12. *Cribrilina annulata*								x			1	1		
13. *Cribrilina punctata*	x				x	x		x			7			
14. *Crisia eburnea*		x	x	x	x	x		x			18	2	8*	Aug. 8±
15. *Cryptosula pallasiana*			x		x	x	x				11	4	16	June–Sept.±

TABLE IV—*Continued*

	Penikese Island, Mass.	New Bedford, Mass.	North Falmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers See under each bryozoan species discussion	Tentacle number. Personal observations unstarred, others starred	In filled ovicell, embryo-producing or larva-releasing stage at Woods Hole
16. *Electra hastingsae*						x	x				2	1		
17. *Electra pilosa*	x	x	x	x	x	x	x	x	x		17	8	12–14	
18. *Flustrella hispida*						x					5	3	13	
19. *Hippoporina contracta*						x					2		12*	
20. *Hippothoa hyalina*	x	x	x	x	x	x		x			17	5	12*–14*	July–Aug.±
21. *Lichenopora hispida*								x			2			
22. *Membranipora lacroixii(?)*							x				3		11	
23. *Membranipora tuberculata*										x	1	3		
24. *Microporella ciliata*					x	x					5	3	13*–14*	Aug.±
25. *Pedicellina cernua*		x	x	x	x	x	x				11	6	8–12; 8*–24*	
26. *Schizoporella biaperta*			x		x	x					9		12	July–Aug.±
27. *Schizoporella unicornis*		x	x		x	x					6	3	about 18*	July–Aug.±
28. *Scruparia ambigua*			x		x						11		10	
29. *Scruparia clavata*					x						1			
30. *Smittina trispinosa*			x		x	x					4			

* The references for the starred* numbers in the Tentacle No. column can be found under each species' descriptive section.

± The plus and minus signs in the last column mean that embryos or larvae very likely were present earlier or later than the days and months listed in the last column.

List of Collected Algae

Chlorophyceae (green algae)

1. *Cladophora gracilis* (Griffiths) Kutzing, forma *tenuis* Farlow
2. *Enteromorpha intestinalis* (Linnaeus) Link (a proliferous form)
3. *Ulva Lactuca* Linnaeus var. *rigida* (C. Agardh) LeJolis

Phaeophyceae (brown algae)

4. *Ascophyllum Mackaii* (Turner) Holmes et Batters
5. *Ascophyllum nodosum* (Linnaeus) LeJolis
6. *Chorda Filum* (Linnaeus) Lamouroux
7. *Cladostephus verticillatus* (Lightfoot) C. Agardh
8. *Desmarestia aculeata* (Linnaeus) Lamouroux
9. *Fucus evanescens* C. Agardh
10. *Fucus vesiculosus* Linnaeus
11. *Fucus vesiculosus* var. *spiralis* Farlow
12. *Laminaria Agardhii* Kjellmann
13. *Laminaria longicruris* De la Pylaie
14. *Sargassum Filipendula* C. Agardh

Rhodophyceae (red algae)

15. *Agardhiella tenera* (J. Agardh) Schmitz
16. *Callithamnion roseum* (Roth) Harvey
17. *Ceramium rubriforme* Kylin
18. *Ceramium rubrum* (Hudson) C. Agardh
19. *Champia parvula* (C. Agardh) Harvey
20. *Chondrus crispus* (Linnaeus) Stackhouse
21. *Corallina officinalis* Linnaeus
22. *Cryptopleura* sp.
23. *Cystoclonium purpureum* (Hudson) Batters var. *cirrhosum* Harvey
24. *Gracilaria confervoides* (Linnaeus) Greville
25. *Gracilaria foliifera* (Forsskål) Børgesen
26. *Lithothamnium* sp.
27. *Phycodrys rubens* (Hudson) Batters
28. *Phyllophora Brodiaei* (Turner) J. Agardh
29. *Phyllophora membranifolia* (Goodenough et Woodward) J. Agardh
30. *Plumaria sericea* (Harvey) Ruprecht
31. *Polyides rotundus* (Gmelin) Greville
32. *Polysiphonia elongata* (Hudson) Harvey
33. *Polysiphonia nigra* (Hudson) Batters
34. *Polysiphonia nigrescens* (Hudson) Greville
35. *Polysiphonia variegata* (C. Agardh) Zanardini
36. *Rhodomela subfusca* (Woodward) C. Agardh
37. *Rhodymenia palmata* (Linnaeus) Greville

Below is a list of the 30 bryozoan species which were found growing on the various algae examined by the authors.

List of Collected Bryozoa

Entoprocta

1. *Pedicellina cernua* (Pallas) 1771

Ectoprocta

Cyclostomata or Stenolaemata

2. *Crisia eburnea* (Linnaeus) 1758
3. *Lichenopora hispida* (Fleming) 1828

Ctenostomata

4. *Aeverrillia armata* (Verrill) 1873
5. *Aeverrillia setigera* (Hincks) 1887
6. *Alcyonidium polyoum* (Hassall) 1841
7. *Bowerbankia gracilis* Leidy 1855
8. *Bowerbankia imbricata* (Adams)1800
9. *Flustrella hispida* (Fabricius) 1780

Cheilostomata

10. *Aetea sica* (Couch) 1844
11. *Bugula cucullifera* Osburn 1912
12. *Bugula flabellata* (Thompson) 1848
13. *Bugula turrita* (Desor) 1848
14. *Callopora aurita* (Hincks) 1877
15. *Cellepora dichotoma* Hincks 1862
16. *Cribrilina annulata* (Fabricius) 1780
17. *Cribrilina punctata* (Hassall) 1841
18. *Cryptosula pallasiana* (Moll) 1803
19. *Electra hastingsae* Marcus 1938
20. *Electra pilosa* (Linnaeus) 1767
21. *Hippoporina contracta* (Waters) 1899
22. *Hippothoa hyalina* (Linnaeus) 1767
23. *Membranipora lacroixii* (?)
24. *Membranipora tuberculata* (Bosc) 1802
25. *Microporella ciliata* (Pallas) 1766
26. *Schizoporella biaperta* (Michelin) 1842
27. *Schizoporella unicornis* (Johnston) 1847
28. *Scruparia ambigua* (d'Orbigny) 1841
29. *Scruparia clavata* Hincks 1857
30. *Smittina trispinosa* (Johnston) 1825

Bryozoan Growths on Algae

The bryozoa form white, grey, yellow. salmon-pink, or brown growths on the algae. Some bryozoan colonies are thin. flat, encrusting and closely adherent. Others are dendritic, arborescent, or may form a fuzzy mass of tiny vesicles. Still others coat the algae with a gelatinous, rubbery, or leathery film. The calcareous

bryozoa often retain their zooecial patterns and specific characteristics pretty well even though the algal host specimens have been dried and pressed in the normal course of herbarium sheet mounting. It was no harder to identify *Hippothoa hyalina* from a dry 1874 herbarium mount of *Phycodrys rubens* than from a freshly collected alga.

Bryozoa grow on various parts of the algal plant. The holdfast processes of Laminaria and related forms are excellent sites for attachment of at least 21 hard, horny, or soft bryozoan species. Bryozoa grow on and between the holdfast processes as well as on the rocks to which the holdfasts adhere. Laminaria and Rhodymenia blades are favorite attachment sites for *Electra pilosa* which is very common and especially abundant on these algae, sometimes coating both sides of the entire blade for an area of several inches with a thin, frosty-white, single-layered cover of contiguous bryozoan colonies. *Membranipora tuberculata* has the same habit of extensively encasing its algal hosts with the fine bryozoan mesh.

The basal or most proximal parts of *Chondrus crispus* and Phyllophora are encrusted by many bryozoans like Aeverrillia, Bowerbankia, *Cellepora dichotoma* and *Hippothoa hyalina,* while the most distal tips are somewhat less often utilized for bryozoan attachment. Sometimes, if the bryozoan growth is especially rich or dense on these two algal genera, the whole blade may be covered. Alcyonidium may encase a whole blade and sometimes extend even beyond the tips of the plant. The two Schizoporellae also may grow so readily as to produce shelf-like extensions of the colony beyond the plant thallus.

The basal parts of Ascophyllum and Fucus are generally favored by the bryozoan colonies, as are the crevices and depressions around the airbladders and where branches originate. *Flustrella hispida* and Bowerbankia particularly favor these plants.

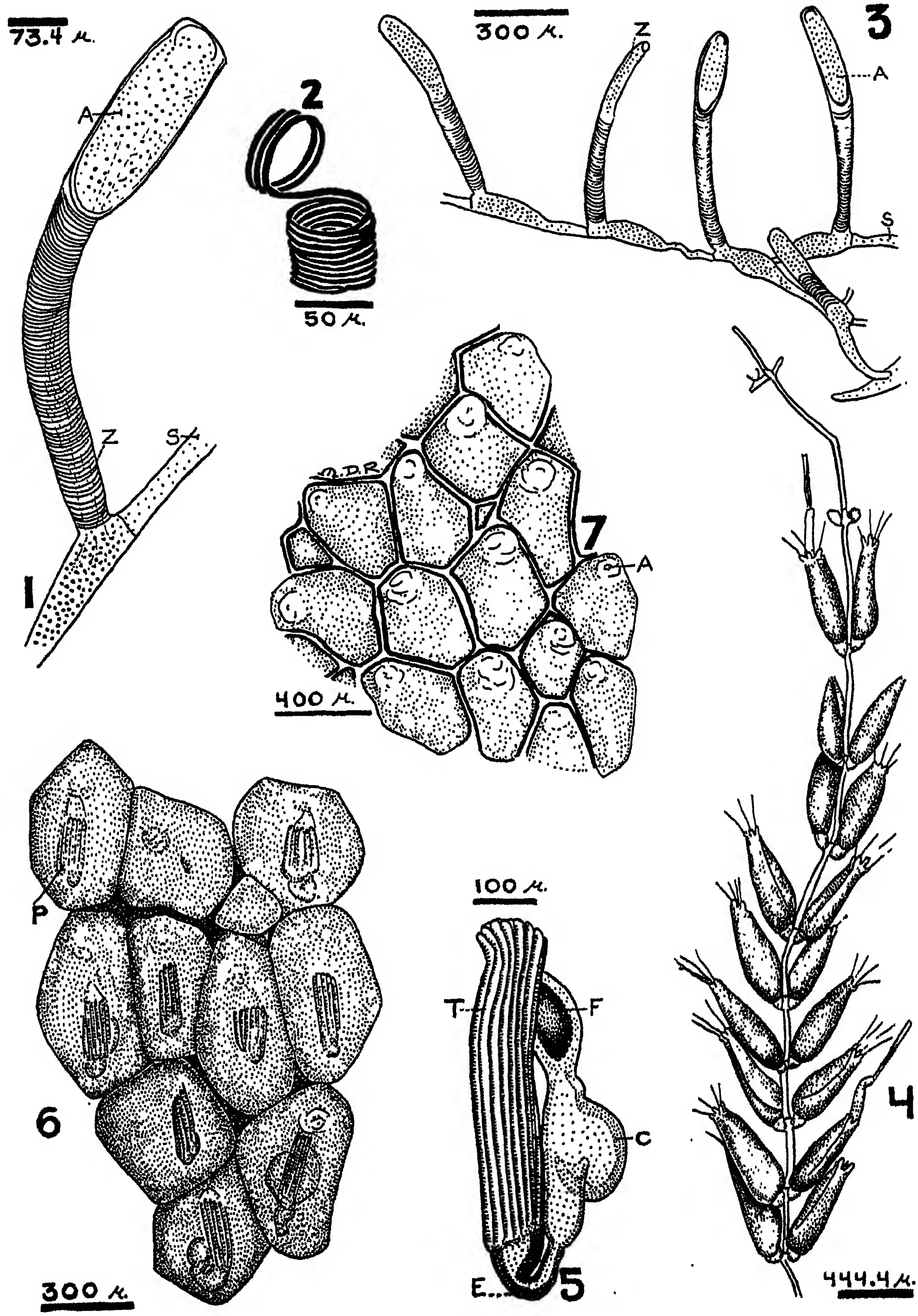
The few zoaria (bryozoan colonies) found on the green algae generally were small, consisting of only a few zoids, and did not produce such luxuriant and extensive growths as did the species which grew on the browns and reds.

The zoaria, as a rule, were one layer in thickness on the algae, with the exception of occasional specimens of *Schizoporella biaperta, S. unicornis* and *Smittina trispinosa,* which might be laminate. The laminate condition is more common on the firmer substrates (rocks) than on algae. *Hippothoa hyalina* and *Electra pilosa* were always single-layered on the plants.

Of the six most frequently encountered bryozoa (*Actea sica, Bowerbankia gracilis* and *Crisia eburnea* each on 18 algal species, *Electra pilosa* and *Hippothoa hyalina* each on 17, and *Bugula turrita* on 16 algal species) the least conspicuous is Actea. It readily escapes detection unless the alga is examined microscopically. Because of their very characteristic growth habit and general appearance, Electra, Bugula, Bowerbankia and Crisia can be recognized with the unaided eye. Hippothoa, with a little practice, becomes recognizable because it forms small, short, calcareous, white sheaths around the thin algal stalks and filaments. Hippothoa especially accommodates itself readily to the smallest filaments and branches.

The bryozoa occurred in close association on the same algal thallus with many other animal forms. Numerous shells of Spirorbis sp. grew alongside the *Lichenopora hispida* from Rye, N. H. Sponges, hydroids, annelid worm tubes, *Botryllus schlosseri, Molgula manhattensis,* Styela, Foraminifera, and several species of bryozoa were sometimes found on a heavily populated alga. Hydroids,

PLATE I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444.4 μ.

Foraminifera, and several bryozoan species often were found on the same blade of Ascophyllum, Chondrus, Laminaria, Phycodrys, or Phyllophora. Aeverrillia and Aetea would sometimes grow on Bugula and hydroid colonies as well as on algal thalli.

The tentacle number and the time of larval production were obtained for some species but not for all because sometimes the colonies died before they could be examined, and sometimes the organisms were so exasperatingly slow in extending their tentacles for a count. Such data as could be obtained are listed in Table IV and also in the species' descriptive section which follows.

Aetea Sica

(Figures 1–3)

Aetea sica is fairly common, although not reported from this region previously. It forms a thin, white, bristly tracery on 18 different algal species. The zoids resemble fine upright tubes just big enough to be seen with the unaided eye. Slender stolons connect the bases of the upright zoids and adhere closely to the substratum (Fig. 3). Nine to eleven tentacles were counted in a few zoids. Ovicells were filled with live developing pinkish larvae from at least July 31 through August 6.

The feature by which Marcus (1937, p. 29) distinguishes *Aetea sica* from the previously reported *Aetea anguina* is the ratio of the aperture (opesium) length to opesium width. The opesial ratio for *A. anguina* is between 1.7:1 and 2:1. For *A. sica* it is between 2.6:1 and 4:1. In Figure 1, one zoid has a 4:1 ratio. If the ratio is a valid characteristic of the two species, then some of the previously reported *Aetea anguina* from the Woods Hole area must belong to *Aetea sica.*

Plate I *

Figure 1. *Aetea sica.* Upright zoid (Z) growing from a punctate stolon (S) enlargement. The opesium (A) of this zoid is about three times as long as wide (a 3:1 ratio). The scale above applies to this figure. Hadley Harbor specimens, VII–28–1939.

Figure 2. *Aetea sica.* Detail of a broken stalk.

Figure 3. *Aetea sica.* A colony of five zoids (Z) arising from stolons (S). The upper right zoid has an opesium (A) about four times longer than wide (4:1 opesial ratio). The scale above applies to this figure.

Figure 4. *Aeverrillia armata.* A sprig of a colony collected from Lagoon Pond, Martha's Vineyard, VIII–17–1945.

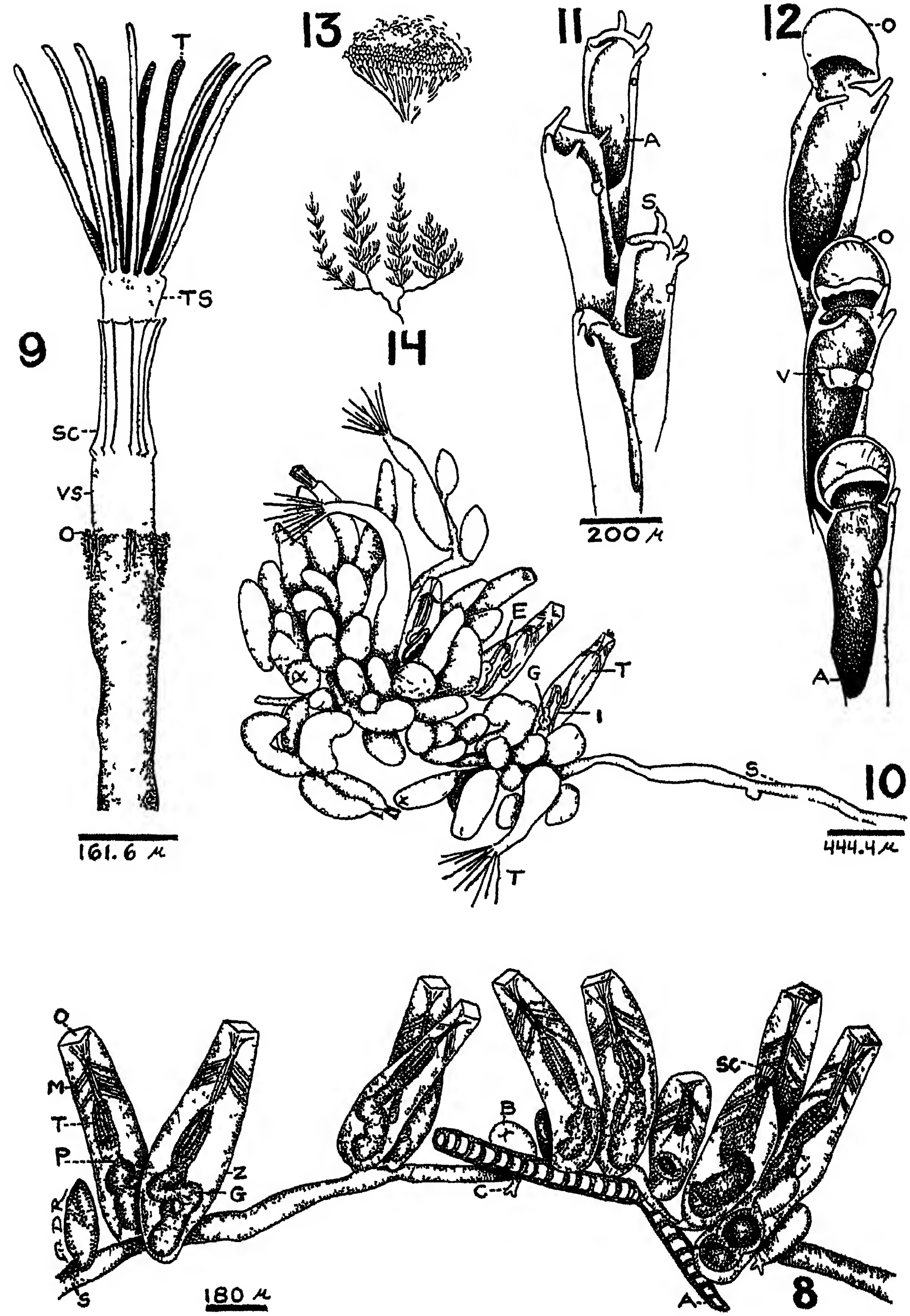
Figure 5. *Alcyonidium polyoum.* A polypide torn out of the colony, in its natural withdrawn position. It consists of tentacles (T), esophagus (E), caecum (C) and rectum which in this sketch contains a large dark fecal pellet (F). Collected off Davenport Park, New Rochelle, N. Y., on IX–22–1945.

Figure 6. *Alcyonidium polyoum.* Part of a young, fairly transparent colony most of whose zooecia contain a sketchily outlined withdrawn polypide (P). Of the same date and collecting locality as specimens of the preceding figure.

Figure 7. *Alcyonidium polyoum.* Somewhat thicker-walled colony, with slightly raised orifices (A). Collected at Black Rock, New Bedford Harbor, on VIII–8–1945.

* Figures on all plates, with the exception of Figures 13, 14 and 28, were drawn with the aid of a camera lucida. The species are alphabetically arranged except for Figure 14.

PLATE II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AEVERRILLIA ARMATA

(Figure 4)

Aeverrillia armata is transparent, yellowish, and horny, and occurs on *Laminaria Agardhii* and *Phyllophora membranifolia*. The latter alga was heavily encrusted with ten other bryozoan species and several algal species. *Aeverrillia armata* consists of numerous slender, paired autozoids arising from narrow stolons which cling closely to the plant but which can be pulled off as slender threads. This species is very similar to *A. setigera* which was discussed very fully in a previous study (Rogick, 1945a), except that it lacks the basal clasping processes of *A. setigera*. The polypides have eight tentacles in both species of the genus.

AEVERRILLIA SETIGERA

This delicate bryozoan was found growing inconspicuously on eight algal species. It clings closely to the plant thallus. It was pictured adequately in the previous study (Rogick, 1945a), so no figure of it is here included. The resemblance between it and *A. armata* is so close that one could easily mistake the one for the other.

ALCYONIDIUM POLYOUM

(Figures 5–7)

The various Alcyonidia are difficult to tell apart. The present *Alcyonidium polyoum* forms a firm gray or sometimes slightly yellowish crust around the hold-

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PLATE II

FIGURE 8. *Bowerbankia gracilis* An uncrowded stolonate colony of nine full-grown zoids (Z) and five smaller buds (B), growing on an algal filament (A). Other structures shown are: (C) caudal process; (G) gizzard, (M) parieto-vaginal musculature, (O) squared orifice; (P) polypide, (S) stolon, (SC) setigerous collar. Collection site and date same as for Figure 7.

FIGURE 9. *Bowerbankia imbricata* Upper part of an extruded polypide showing ten tentacles (T) which upon retraction can be withdrawn into the tentacular sheath (TS). Around that is a stiff transparent setigerous collar (SC) which in turn can be withdrawn into the vestibular sheath (VS). Some debris has accumulated on the edge of the squared orifice (O). From Glen Island, New Rochelle, N. Y. on IX–16–1945.

FIGURE 10. *Bowerbankia imbricata* A crowded colony which was scraped from *Chondrus crispus*. Collection date and site the same as for the preceding figure Three of the long zoids have their tentacles extended. Three smaller ones have their setigerous collars partly extruded. Three zoids are shown with the polypides within them. The following parts are labelled: (E) esophagus, (G) gizzard; (I) intestine; (S) stolon; (T) tentacles

FIGURE 11. *Bugula cucullifera.* Four zooecia, each provided with four spines (S). The upper three zoids show, at the side of the opesium (A), the remains of the short peduncle which had borne an avicularium. From Provincetown, Mass., on VIII–18–1947; Dr. M. Doty collector.

FIGURE 12. *Bugula cucullifera.* Three fertile zooecia topped with ovicells (O). The middle zooecium bears an avicularium (V), the other two have lost theirs. (A) is the opesium. The second row of zooecia which normally would be at the side of these zooecia was incomplete and was therefore not shown here. Same collection date and site and drawn to the same scale as the preceding figure.

FIGURE 13. *Bugula flabellata.* A freehand sketch, showing the close tuft-like mode of colony growth. About natural size.

FIGURE 14. *Bugula turrita.* A freehand sketch, showing the dainty, open spiral mode of colony growth. About natural size.

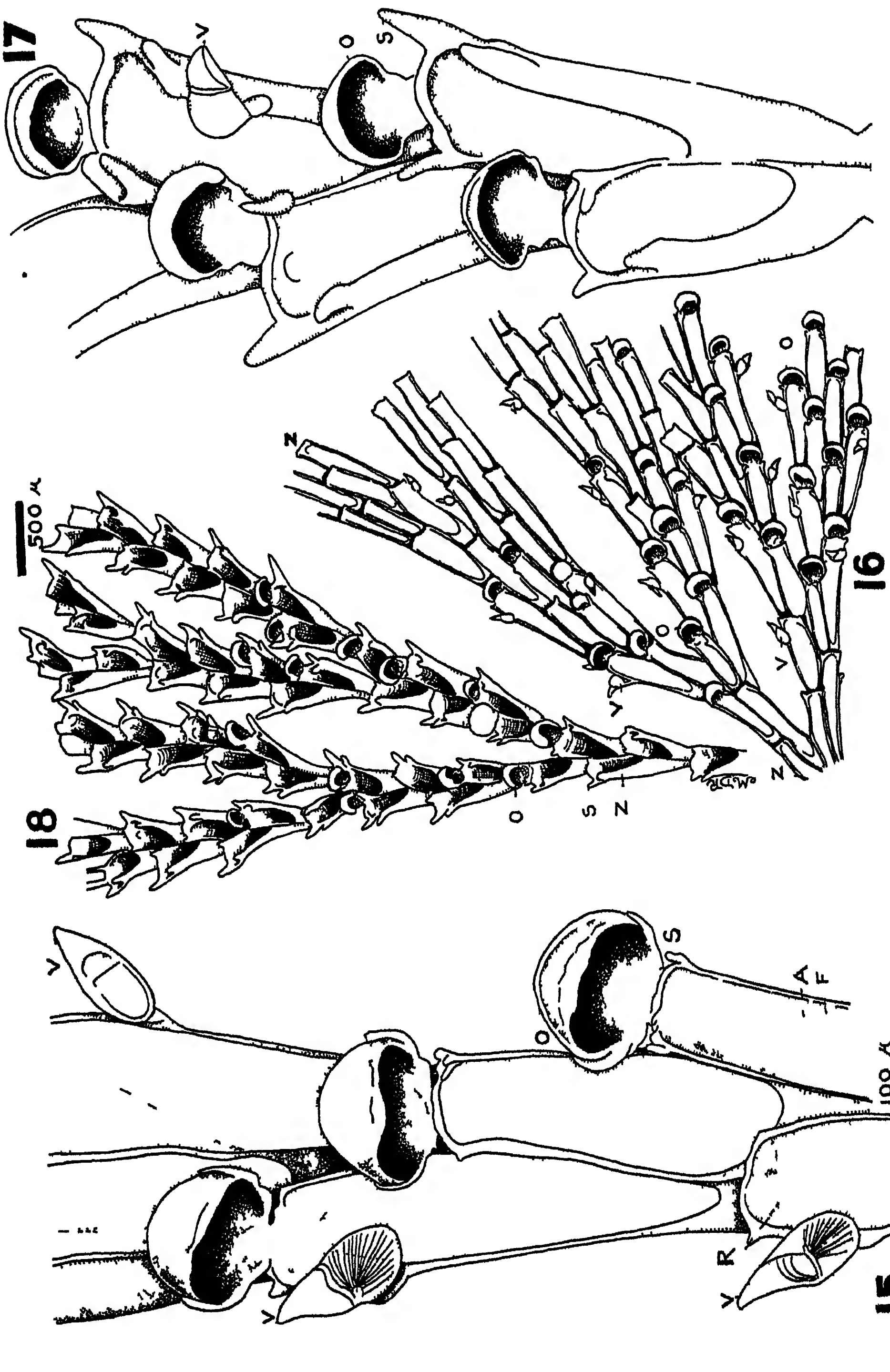


PLATE III

fasts, stalks and blades of at least five algal species Prenant and Teissier (1924, pp 23, 27) reported Alcyonidium from Ascophyllum Chondrus, Fucus, Himanthalia *Laminaria* (*saccharina?*) and *Saccorhiza bulbosa* Sometimes it coats the entire alga, using the various branches as cores around which to grow The colony is rubbery to the touch

The polypides had 16 tentacles Measurements for 18 zoids ranged thus zoid length 0 36–0 648 mm and zoid width 0 24–0 504 mm These are similar in range to figures given by Harmer (1915, pp 37–38) The extremes in tentacle number given by various authors are 12 (Harmer 1915, p 38) to 20 (Silén, 1942, p 11)

Bowerbankia Gracilis

(Figure 8)

Bowerbankia gracilis is very common It forms a soft grayish furry mass on 18 algal species It consists of a number of transparent tubes clustered along a stolon sometimes so densely that the stolon is scarcely visible Caudal processes appear on some zooecia The eight tentacles can be counted only when the animal is alive and in the expanded state The zoids in Figure 8 are in the retracted state with the tentacles and gut (collectively called polypide") withdrawn into the body cavity Under such conditions the squared orifices show nicely

Bowerbankia Imbricata

(Figures 9–10)

Bowerbankia imbricata was found on only five algal species by the authors Additional species on which it has been reported are *Ascophyllum nodosum* (Adams, 1800, p 11) *Corallina officinalis* (Hincks, 1880, p 521), *Cystoseira fibrosa, Fucus serratus* (Joliet 1877, p 294) *Desmarestia aculeata,* and *Furcellaria fastigiata* (Thompson 1840, p 252) Colonies may cover extensive areas of several inches coating the "stems" and thalli of Chondrus They do not exclude other forms from growing on the alga but may grow among hydroids, sponges, and other encrusting forms

Superficially, dense growths of *Bowerbankia imbricata* and *B gracilis* are indistinguishable Imbricata colonies whose zoids were filled with large ciliated

Plate III

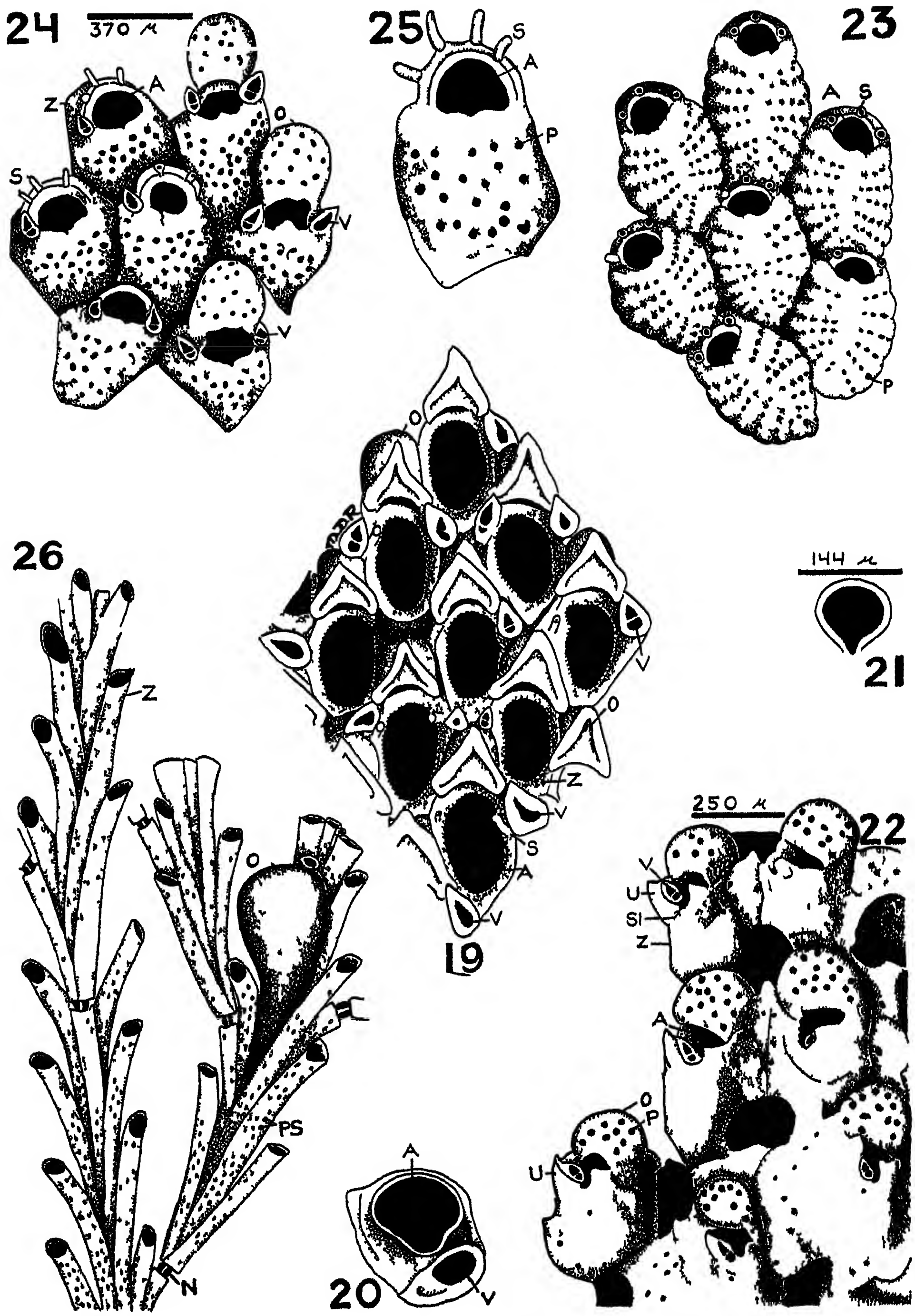
Figure 15 *Bugula flabellata* The upper parts of three fertile ovicell bearing zooecia (F) and an ordinary zooecium (R) Other structures shown are (A) opesium, (O) ovicell, (S) spine, (V) avicularium These same labels apply to the other figures on this plate

Figure 16 *Bugula flabellata* Broad flabellate branches with up to 6 rows of zooecia (Z) per branch Some have ovicells, some avicularia, or both, and others have neither, at the moment Drawn to the same scale as Figure 18

Figure 17 *Bugula turrita* Four fertile zooecia, each topped by a very shallow, fragile ovicell, set at an angle on the upper edge of the zooecium Spines are well developed in this colony and one avicularium is shown Drawn to the same scale as Figure 15 Collected at Woods Hole, VI–30–1938

Figure 18 *Bugula turrita* Branches showing biserial arrangement of zooecia (Z) Some zooecia bear ovicells (O)

PLATE IV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globular larvae were salmon pink in color because the red pigment of the larvae showed through the parent zoid walls. Such embryos were especially abundant in colonies collected during the first ten days in August (1947). Some embryos were found in colonies collected as late as August 31. Joliet (1877, p. 295) observed larvae during the month of July and reported that sexual reproduction took place from the end of June to early August.

Somewhat reniform larvae were released in great numbers on the morning of August 9, 1947. After a free swimming period they attached to the substratum. Metamorphosis proceeded speedily, taking less than five minutes in some cases. The red color became concentrated at one end of the metamorphosing larva.

Adult zoids generally have ten tentacles and are square topped when retracted. Measurements of seven retracted zoids were as follows: zoid length 0.925–1.374 mm; zoid width 0.178–0.291 mm; stolon diameter 0.040–0.101 mm.

Some very young colonies consisting of only one or two developing zoids had nine tentacles but their development could not be followed beyond a few days, so it could not be determined if these in time would increase their tentacular number to ten.

BUGULA CUCULLIFERA

(Figures 11–12)

Small fragments of this Bugula were found on *Fucus evanescens* and *Rhodymenia palmata* from Vineyard Sound on VIII–1–1945; on *Laminaria longicruris* from Provincetown, Mass., on VIII–18–1947 and on *Phyllophora membranifolia*, along with much *Crisia eburnea* and *Aetea sica* from New Bedford Harbor, on VIII–8–1945.

PLATE IV

FIGURE 19. *Callopora aurita*. Nine zooecia (Z) each capped by a rounded ovicell (O) which is decorated by a raised triangular ridge. Spines (S) and avicularia (V) are present near the large opesia (A). The same letters apply to the other figures on this plate. A calcined specimen from which all the soft tissues have been burned away. Drawn to the same scale as Figure 24.

FIGURE 20. *Cellepora dichotoma*. Part of a very lightly calcified specimen showing the shape of the aperture and the position of the avicularium. From Nobska Beach driftweed, Woods Hole, VII–25–1944. Drawn to the same scale as Figure 21.

FIGURE 21. *Cellepora dichotoma*. Aperture of a very young zooecium.

FIGURE 22. *Cellepora dichotoma*. Portion of a moderately calcified colony. An avicularium is borne on the side of the umbo (U) and faces toward a sinus (SI) in the peristome or raised shelf encircling the front of the aperture. The ovicells have pores (P).

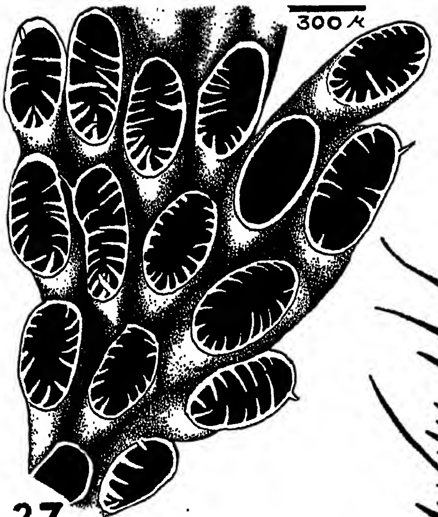
FIGURE 23. *Cribrilina annulata*. Seven zooecia. Drawn to the same scale as Figure 24.

FIGURE 24. *Cribrilina punctata*. Seven zooecia, three of which are capped by ovicells. One or two avicularia border the wide aperture. From Penikese Island, Mass., VIII–3–1947, on *Chondrus crispus*.

FIGURE 25. *Cribrilina punctata*. A more heavily calcified zooecium with 4 spines above the aperture. From Gay Head, Martha's Vineyard, VII–30–1946. Drawn to same scale as Figure 21.

FIGURE 26. *Crisia eburnea*. Four internodes separated by dark yellow horny joints or nodes (N), bear a number of tubular autozoids (Z). One internode bears the greatly swollen ovicell or ooecium. The zooecia have numerous pseudopores (PS). From Black Rock, New Bedford Harbor, VIII–8–1945. Drawn to same scale as Figure 24.

Plate V



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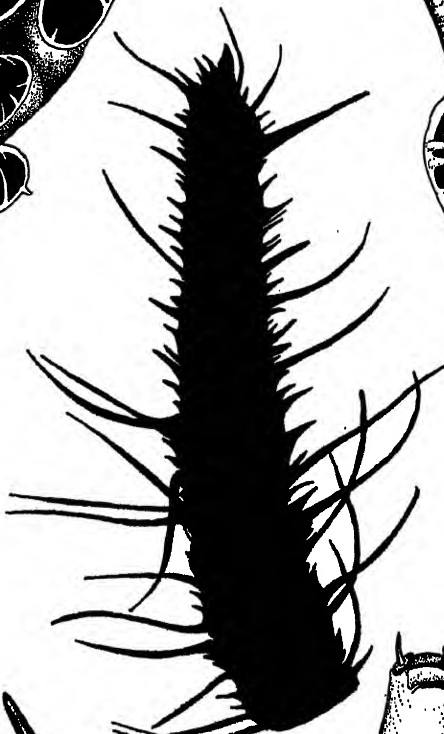

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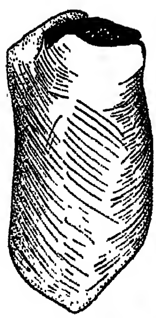

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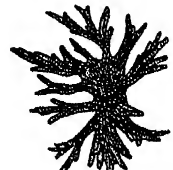

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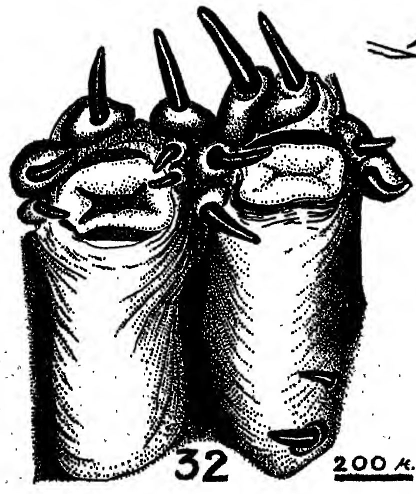

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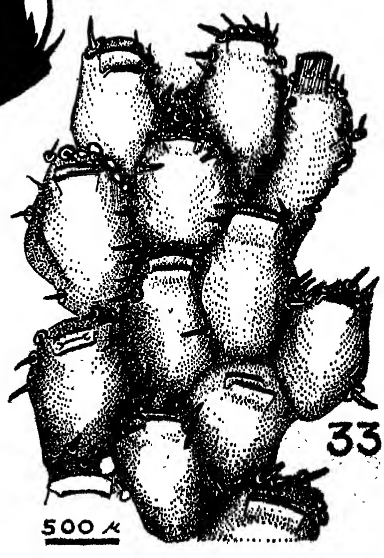


33

Some embryo-filled ovicells were present. Very long rhizoid processes grew from the basal part of some of the colonies. Thirteen tentacles were counted on one zoid.

Bugula Flabellata

(Figures 13, 15, 16)

A small colony of *B. flabellata* was found on *Fucus vesiculosus*. It was far less common than *B. turrita*. Also, it seemed to prefer attachment to piles, live cars, and other submerged wooden objects rather than to algae. It is a very sturdy form, growing in thick, fan-shaped, yellow-orange tufts (Fig. 13) which are about a half inch tall.

Glass slides submerged in Eel Pond at Woods Hole from August 13 to August 31, 1945, were heavily overgrown with various animal forms, including *Bugula flabellata*. Colonies of the latter were by then about ¼ inch tall and contained hundreds of zoids.

According to Grave (1933, p. 384) its breeding season is between June 1 and November 15.

Bugula Turrita

(Figures 14, 17, 18)

Bugula turrita is very common, growing on at least 16 algal species. In general appearance it is more plant-like than animal-like. It is of yellow-orange color and has a soft, fluffy, but firm texture. It has a beautifully spiralling manner of growth (Fig. 14). The colony branches into a number of spiralling "turrets." Some of the colonies may be 1¼ inches tall.

The tentacle number is about 14.

Ovicells were seen in colonies collected from the end of June through mid-August (Fig. 17). Many young colonies developed from released larvae during that time.

Plate V

Figure 27. *Electra hastingsae*. Fifteen zooecia from the central part of a colony. One zooecium has lost all the spines around its opesium. The others have retained a varying number. Calcined specimen.

Figure 28. *Electra hastingsae*. A freehand sketch showing the flat, spray-like mode of growth which is so characteristic of this species. About natural size.

Figure 29. *Electra pilosa*, long-spined form. Tip of an alga, *Desmarestia aculeata*, completely encased by a bryozoan colony some of whose zooecia show an unusually long median spine. Shown in silhouette. Collected off Gay Head, Martha's Vineyard, VII–30–1946. Drawn to the same scale as Figure 33.

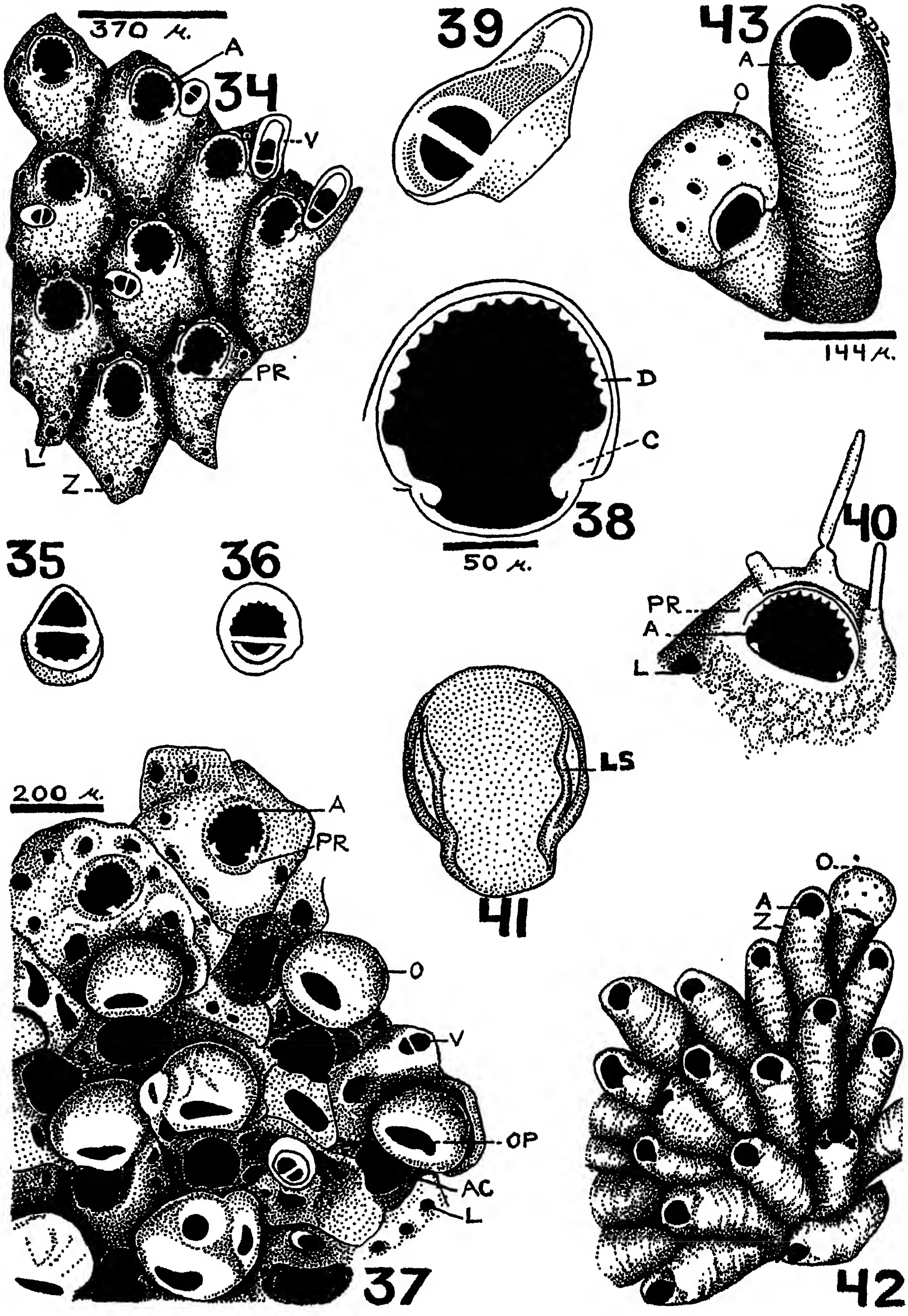
Figure 30. *Electra pilosa*, short-spined form. Four zooecia whose lowest, median spine is heavier and longer than the other opesial spines but not so long as the spines pictured in Figure 29. The two upper zoids show the crescent-shaped operculum rim in the upper part of the opesial area. The lower frontal wall of the zooecium is marked by numerous tremopores. From Devil's Foot, Woods Hole, VII–9–1945.

Figure 31. *Flustrella hispida*. A very young, spineless zoid. From Woods Hole, VIII–15–1939. Drawn to same scale as Figure 32.

Figure 32. *Flustrella hispida*. Two old zoids showing heavy "chitinization" of spines and lips of the orifice. The left zoid shows only circumoral spines while the right shows those and also additional spines located lower down on the zoid. From same colony as Figure 31.

Figure 33. *Flustrella hispida*. Twelve spine-encircled zoids from a less heavily "chitinized" part of the same colony as Figure 32.

PLATE VI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200 μ.
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37
O
A
Z
42

Callopora Aurita

(Figure 19)

Callopora aurita was not abundant on algal material, being found more commonly and in more extensive patches on rocks. Very small white colonies were found on specimens of *Phycodrys rubens* collected from Rye Ledge, Rye, New Hampshire, on IX–30–1945; on *Phyllophora membranifolia* dredged from Great Harbor, Woods Hole, Mass., on VIII–8–1946, and on holdfasts of *Laminaria Agardhii*. The colonies form a fine encrusting calcareous mesh on the algal thallus.

Ovicells were present in the colonies, but it was not possible to determine whether they were tenanted by larvae at the time of collection. Twelve tentacles were counted on one zoid.

Cellepora Dichotoma

(Figures 20–22)

Its small, white, calcareous zoaria grow on *Chondrus crispus, Gracilaria confervoides, Phyllophora Brodiaei* and *P. membranifolia*. Its zoids are crowded

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Plate VI

Figure 34. *Hippoporina contracta.* Portion of a young, uncrowded colony showing nine zooecia (Z), four of which are without avicularia (V) and three of which have a small rounded avicularium and two of which have spatulate avicularia (V). The distinctive serrate aperture (A) is readily distinguishable in these not heavily calcified zoids. The peristome (PR) is prolonged into a small bump or mucro below the aperture in the central zoid. Areolae (L) border each zooecium. The same labels apply to the other figures on this plate. Specimens dredged from Great Harbor, Woods Hole, on VIII–8–1946.

Figure 35. *Hippoporina contracta.* A small, slightly pointed avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

Figure 36. *Hippoporina contracta.* A small rounded avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

Figure 37. *Hippoporina contracta.* A more crowded and calcified colony than that of Figure 34. The upper two zoids show the typical serrate aperture. Seven ovicells (O) with large, comma-shaped pores (OP) are visible. Beneath each ovicell is a large peristomice (AC) or peristomeal opening (here shown in black), at the bottom of which lies the distinctive serrate aperture (invisible in this picture). Calcined specimen.

Figure 38. *Hippoporina contracta.* The serrate aperture characteristic of this species. The aperture, black in this calcined specimen, in life is closed over by an operculum which is pictured in Figure 41. The aperture has 14 to 18 small rounded denticles (D) and two large bifid cardelles (C).

Figure 39. *Hippoporina contracta.* A spatulate avicularium seen at an angle. Drawn to the same scale as Figure 43.

Figure 40. *Hippoporina contracta.* Upper half of a zooecium showing areola (L), denticles, cardelles, peristome (PR) and three spines above the aperture (A). Drawn to same scale as Figure 43.

Figure 41. *Hippoporina contracta.* Operculum which closes the aperture of the zooecium. It has a stiffened rim and lateral sclerites (LS). Drawn to same scale as Figure 38.

Figure 42. *Hippothoa hyalina.* A colony showing a number of ordinary zooecia (Z) and a dwarfed one topped by an ovicell (O). Drawn to same scale as Figure 34.

Figure 43. *Hippothoa hyalina.* Another view of the punctate ovicell, its dwarfed zooecium and a normal sized zooecium. The latter shows the typical aperture, rounded and with a sinus. The transverse grooving normally found in the zooecia is faintly indicated in the larger zoid. Specimens dredged off Gay Head, Martha's Vineyard, VII–30–1946.

against each other. Embryo-filled ovicells were present at the time of collection (July 25, 1944).

There is some question as to the classification of this species. *Cellepora americana, Cellepora avicularis* and *Cellepora dichotoma* show such integradation that their exact status or validity needs critical review by some future worker. The species of the present study is identical with Marcus' illustration of *C. dichotoma* (Marcus, 1938, Plate XI, Fig. 26).

The species characteristics are as follows: (1) peristome with a sinus next to a raised umbo on the side of which is an avicularium facing the sinus; (2) aperture rounded, with postral sinus; (3) ovicell with pores, rounded and somewhat flattened; and (4) a few small pores (areolae) around the frontal wall of the zooecium.

A heavily calcified zoarium may show the ovicells almost completely immersed on all sides except the frontal in the secondarily calcified zooecial wall. The frontal of such ovicells is provided with good-sized pores and is at a lower level than the secondarily calcified outer zooecial wall.

Cribrilina Annulata

(Figure 23)

This encrusting species was very uncommon. Only one white calcareous zoarium was found on *Phycodrys rubens,* from Rye, N. H., on the reverse side of the thallus from the finer, more fragile *Cribrilina punctata.* Three or four spines were present around the aperture. Marcus (1940, p. 203) reported *C. annulata* from Laminaria.

Cribrilina Punctata

(Figures 24–25)

Small patches of this fragile white calcareous form were found encrusting seven algal species. The number of spines around the aperture varied from none to five. The frontal pores were somewhat irregular in size and position. This was not a very common form; only a few colonies appeared in the collection.

Crisia Eburnea

(Figure 26)

Crisia eburnea was exceedingly common on 18 algal species. It was especially abundant on *Chondrus crispus,* the two Phyllophorae, and *Phycodrys rubens.* A very large amount of it was collected from the driftweed along the beaches at Nobska, Gay Head, and Cuttyhunk. Dried specimens were just as useful as wet ones for taxonomic purposes.

Prenant and Teissier (1924, p. 18) reported *C. eburnea* from Halidrys and certain Cystoseiras.

It forms brittle, white, openly dendritic tufts up to 7 mm. tall on the thalli of the small, and around the holdfasts of the large, algae. A Crisia colony consists of a number of calcareous tubular zooecia forming internodes which are separated

from other internodes by short, narrow, yellowish to brown chitinous joints. A branch which consists of ordinary tubular zooecia (autozoids) alone is called a sterile internode. Three such are pictured in Figure 26. A branch which consists of a number of autozoids and a long, very swollen brood chamber (ooecium or ovicell) is called a fertile internode. One is pictured in Figure 26. In the identification of different species of Crisiidae the number of zooecia in the fertile and sterile internodes is important. In *Crisia eburnea* the sterile internode has four to eleven zooecia, and a fertile internode seven to ten (Borg, 1944, p. 158).

Ovicells were found on specimens collected throughout the summer months. Embryos were seen in some on August 8, 1946.

Cryptosula Pallasiana

Cryptosula pallasiana forms a round, flat, regularly patterned, pale orange to white encrustation on rocks, shells, and algae. It occurs more commonly and forms larger colonies on the harder substrates than on the algae but is not uncommon on the latter. It was found on eleven algal species which came from a number of collecting sites between Martha's Vineyard, Woods Hole, North Falmouth, and New Bedford (all in Massachusetts). They grew on the thalli of algae and on the Laminaria holdfasts. Colonies attached to *Enteromorpha intestinalis* and *Ulva Lactuca* var. *rigida* were young and small, consisting of few (five or less) freshly formed zoids (as of VIII–13–1945). Submerged glass slides, left in Eel Pond at Woods Hole for the first two weeks in July and kept a week longer in running sea water in the laboratory, were well covered with many animal forms including *Bugula turrita, Pedicellina cernua* and *Cryptosula pallasiana.* The Cryptosula colonies had from one to thirty zoids on these slides. Their polypides had 16 tentacles.

Barrois (1877, p. 139) reported larvae in August and September. The Woods Hole specimens produced larvae in those months as well as during June and July.

Joliet (1877, p. 291) reported this bryozoan on *Callothrix pannorum.* Prenant and Teissier (1924, p. 23) reported Cryptosula from other Laminariae, Himanthalia, and *Saccorhiza bulbosa.*

No drawings of Cryptosula are here included because the species was previously figured (Rogick, 1945b, p. 3, Fig. 1).

Electra Hastingsae

(Figures 27–28)

A few small colonies of *E. hastingsae* encrusted the thalli of *Fucus vesiculosus* var. *spiralis* and *Laminaria Agardhii.* Marcus (1938, p. 17) reported the bryozoan from Zostera. Sometimes it grows on the gill chamber of Libinia crabs. Generally, however, the bryozoan is found on hard substrates (rocks and shells) more often than on algae.

Electra hastingsae is a fragile, white, calcareous species, forming completely adherent frond-like traceries on the substratum (Fig. 28). Some colonies lack spines around the opesium. Other colonies have a variable number of very delicate ones, sometimes as many as 18. Some of the spines may break off (Fig. 27).

A new zooecium may occasionally grow right out of the opesium of another empty one. Whether that is a case of regeneration or the settling of a new larva on an old colony, is not certain. Embryos were not observed.

Electra Pilosa

(Figures 29–30)

Electra pilosa is an extremely common calcareous but fragile encrustation on 17 algal species. It has been reported previously from: *Fucus serratus* (Joliet, 1877, p. 290); Ulva (Hutchins, 1945, p. 540); *Laminaria saccharina* (Leidy, 1855, p. 9); Furcellaria and Polyides (Marcus, 1940, p. 118); the Cystoseiras, Corallina (Prenant, 1927, p. 24) and Zostera (Prenant, 1932, p. 92).

Electra pilosa forms grayish-white, single-layered colonies which spread like a fine, closely-woven mesh over large areas, sometimes a foot in length, of algal thalli. Laminaria and Rhodymenia thalli are particularly favored. Numerous colonies may grow toward and into each other to form an almost continuous thin crust over the thalli. The lacy *Plumaria sericea* fronds, in some instances, were completely encased in *Electra pilosa.* Many Foraminifera were scattered over the Electra.

Great variation in degree of spination occurs. Several *E. pilosa* "forms" of dubious validity are mentioned in literature: forma *typica*, f. *dentata*, f. *laxa* and f. *verticillata*, differing slightly from each other, mainly in the presence or length of the principal median proximal spine. Borg (1930, p. 63) and others mentioned that occasionally several of these growth forms may be found in a single *E. pilosa* colony, and therefore should not be considered valid varieties.

The present writers found both long-spined (forma *verticillata*, Fig. 29) and short-spined (forma *dentata*, Fig. 30) growths in the collections, the latter being far more common than the long-spined specimens.

Tentacles numbered 12 to 14.

Flustrella Hispida

(Figures 31–33)

Flustrella hispida grows on five Woods Hole algal species: *Ascophyllum nodosum*, *Chondrus crispus*, *Fucus vesiculosus*, *Phyllophora membranifolia* and *Ulva Lactuca* var. *rigida*. Also, the M. B. L. Collecting Crew has on numerous occasions brought in Ascophyllum covered with Flustrella from other localities. Additional algae from which it has been recorded are: *Gigantina mamillosa* (Hincks, 1880, vol. 1, p. 507); *Fucus serratus* and Cystoseira (Joliet, 1877, p. 292). It was far more common on Ascophyllum and Fucus than on the green or red algae in the Woods Hole region.

Flustrella hispida forms a brown, rubbery, and somewhat slimy crust over extensive areas of the algal thallus. The zoids are fairly soft and baggy (Figs. 31, 32). Thirteen tentacles were counted on one specimen. Spines were lacking in the very youngest zoids (Fig. 31), but more mature ones show variation in distribution and number of spines (Figs. 32, 33). In the oldest parts of the colony the spines may become very thick and dark reddish brown, and appear mounted on horny pads (Fig. 32) about the zooecial orifices. In younger colonies, and also in some older zooecia, as in the right zoid of Figure 32, spines appear elsewhere about the zoid than just around the orifice. The reinforced orifices are shaped like the top of a purse (Figs. 32, 33).

Barrois (1877, p. 214) found *F. hispida* colonies filled with embryos during the months of May, June and July.

Hipporina Contracta

(Figures 34–41)

White to buff-colored colonies of this species were found more often on rocks and shells than on algae. However, some did grow on *Phyllophora Brodiaei* and *P. membranifolia,* and were up to 2 cm. in diameter. The appearance of the colony varies greatly, depending upon the age of the colony, degree of calcification, the presence of ovicells and the nature of the substratum (compare Figs. 34 and 37).

The key character in identifying this species is the "beaded" aperture (Fig. 38) whose circular outline, serrate antral border, and two bifid cardelles marking the postral border vary so little that they can be identified in either old or young colonies. The number of rounded denticles in the antral border ranges from 14 to 18. In old, heavily calcified colonies (Fig. 37), the zooecial wall and peristome around the aperture may increase in thickness so greatly that the primary "beaded" aperture comes to lie considerably below the external body wall surface, at the bottom of a calcareous "well," the wall of which is formed by the peristome. The top opening of this calcareous "well" is called either the secondary aperture or the peristomice (Fig. 37, AC).

In younger, less calcified colonies, two to six oral spines, sometimes measuring 0.12–0.13 mm., may appear on the peristome (Fig. 40). These break off and their bases may become completely overgrown in the process of increasing calcification of the body wall.

Marcus reported 12 tentacles for this species (1937, p. 98).

The ovicells are quite characteristic also. They are smooth, hemispherical, and provided with a large, comma-shaped membranous area or pore (Fig. 37, OP) on the frontal surface.

Six to thirteen marginal pores or areolae (Fig. 34, L) can be seen in the zooecial body wall.

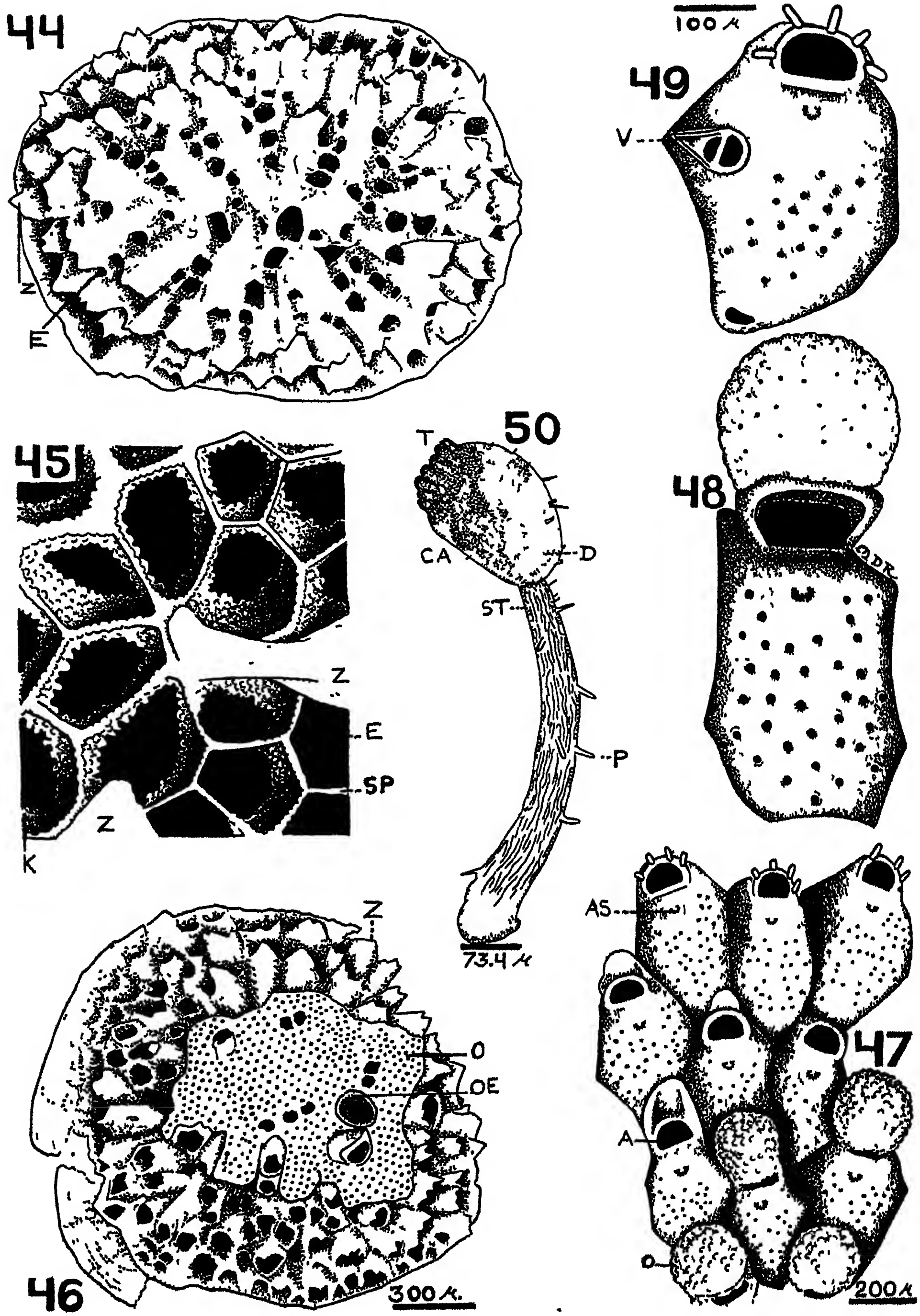
Hippothoa Hyalina

(Figures 42–43)

Hippothoa hyalina was extremely common on 17 algal species in the Woods Hole region. Borg (1930, p. 84) listed it from *Laminaria saccharina;* Prenant and Teissier (1924, p. 22) from the Florideae and Dictyota, and Prenant (1927, pp. 26–27) from *Laminaria cloustoni* and *Saccorhiza bulbosa.* A dry herbarium mount of a pressed *Phycodrys rubens* (collected by Dudley at Marble Head in Sept. 1874) was examined by the writers and found to contain easily recognizable and uncrushed *H. hyalina* zoaria.

Hippothoa hyalina forms glistening white or grey calcareous patches usually from 1 to 8 mm. in diameter, either on or encircling the thalli of most of the mentioned algae and on the holdfasts of Laminaria and Rhodymenia. "Stems" or filaments of *Cystoclonium purpureum* var. *cirrhosum* were encased in rough

PLATE VII



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calcareous sheaths of *H. hyalina* sometimes an inch in length. Often the sheaths of colonies were arranged in a linear series, the total series attaining a length of several inches.

Embryo-filled ovicells were plentiful in specimens collected during July and August in the Woods Hole area. Many ancestrulae or the single individuals from which a colony begins were observed in collections made up to August 11, 1945. These ancestrulae arise from sexually produced larvae. Barrois (1877, p. 164) remarked that at Roskoff the embryos were carried in transparent ovicells in the months of May and June, so apparently the breeding season is of considerable length.

Lichenopora Hispida

(Figures 44–46)

One fertile and less than a dozen small immature colonies of this species were found growing on *Phycodrys rubens* and *Phyllophora membranifolia* collected from Rye, N.H. on IX–30–1945.

The fertile colony (Fig 46) has a brood chamber provided with a thin, rounded aperture and many small pores. The autozoids (Fig 44) terminate in jagged edges They are partly surrounded by reticulate alveoli (Fig. 45) which are lined with small calcareous projections from the interalveolar septa whose thickness is variable.

Borg (1926) gives a good account of the development of various Cyclostomata, including the Lichenoporae, and discusses the terminology of the group.

Membranipora Lacroixii (?)

Membranipora lacroixii is a species whose identification and synonymy are exasperatingly confused in literature. Part of this is due to vague original

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Plate VII

Figure 44 *Lichenopora hispida* A fairly young colony showing autozooecia (Z) separated by large cavities or alveoli (E) Drawn to same scale as Figure 46.

Figure 45 *Lichenopora hispida.* Detail of the center of an immature though fair-sized colony, showing about 17 alveolar spaces (E), the interalveolar septa (SP) between them and the projections (K) from the calcareous cryptocyst of the septal wall. The sides of two autozoids (Z). Drawn to same scale as Figure 49.

Figure 46 *Lichenopora hispida.* A damaged, fertile colony, showing an irregular, punctate brood chamber (O), the brood chamber aperture (OE) and numerous short (immature or damaged?) and some normal autozoids (Z).

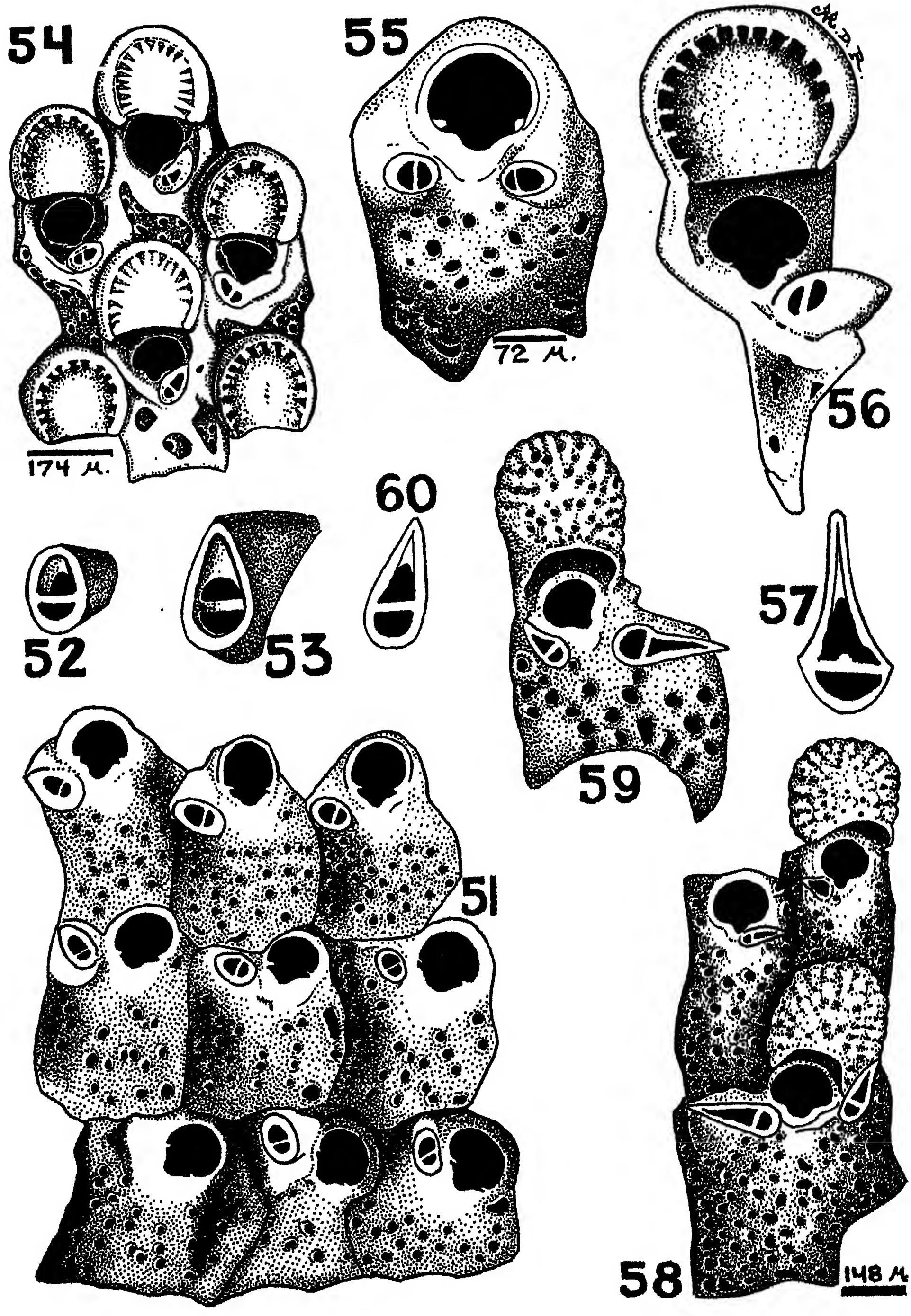
Figure 47. *Microporella ciliata.* Nine zooecia, four of which have well-developed ovicells (O) and three of which have shelf-like beginnings of ovicells distal to the aperture (A). Each zooecium has a crescent-shaped ascopore (AS) and smaller frontal pores. Oral spines occur on the upper three zoids The crescent-shaped ascopore and the hemispherical aperture are key characters for this species

Figure 48. *Microporella ciliata.* A zooecium topped by an ovicell. Both have pores but of different size. Drawn to same scale as Figure 49.

Figure 49. *Microporella ciliata.* A zooecium showing oral spines and a pointed avicularium (V) in a characteristic position. The avicularium may develop on either the right or left side of the zoid. Same collection area and date as Figure 43.

Figure 50. *Pedicellina cernua.* A single zoid consisting of a stalk (ST) and a calyx (CA) containing the polypide (D) and rolled in tentacles (T). A few spines (P) occur on the stalk and calyx. From Black Rock, New Bedford Harbor, VIII–8–1945.

PLATE VIII



descriptions, and part due to the apparently great variation in spination and degree of calcification of the zooecia. Such will continue to be the state of affairs until someone takes the trouble to make a very elaborate study of the variations of this species. The Woods Hole and New Rochelle specimens of the present study resemble the *Membranipora lacroixii* pictured by Osburn (1912, Plate 22, Fig. 22), the *Conopeum lacroixii* pictured by Canu and Bassler (1920, Plate 13, Fig. 9), the *Conopeum reticulum* pictured by Harmer (1926, Plate 13, Fig. 12), the *Biflustra aciculata* of MacGillivray (1891, Plate 9, Fig. 5) and the *Membranipora crustulenta* of Osburn (1944, Fig. 20, p. 32). Our specimens differ from the *Conopeum reticulum* pictured by Marcus (1938, Plate 2, Fig. 5A), the *Conopeum lacroixii* figured by Canu and Bassler (1923, Plate 29, Fig. 4), the *Membranipora reticulum* f. *lacroixii* and *M. crustulenta* of Borg (1930, pp. 63–65). The present study specimens definitely are not the *M. crustulenta* of Borg because that species is pictured with a calcified operculum, a character not present in our specimens. Until the status and limits of the species are fixed, the present authors will continue to call it *M. lacroixii,* as in Osburn's 1912 paper.

Membranipora lacroixii was found encrusting rocks, shells, and less frequently the algae *Ascophyllum Mackaii, Chondrus crispus* and *Phyllophora membranifolia*. It formed a delicate, gray-white tracery which adhered so closely to the substratum, especially rocks, that it was difficult to dislodge. No avicularia or ovicells were found. The conspicuous triangular spaces mentioned as characteristic by Harmer and Marcus were not observed on our specimens. Calcifica-

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PLATE VIII

FIGURE 51. *Schizoporella biaperta.* Nine regularly arranged, moderately calcified zooecia, one of which is without an avicularium. Drawn to the same scale as Figure 54. Calcined specimen.

FIGURE 52. *Schizoporella biaperta.* A small ellipsoidal avicularium. Drawn to same scale as Figure 55.

FIGURE 53. *Schizoporella biaperta.* A small oval or somewhat pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 54. *Schizoporella biaperta.* A fertile area of a colony showing six ovicells. The middle ovicell is most nearly typical in appearance. The frontal area of its zooecium is more highly calcified than that of the other three zooecia above it, and than that of the zooecia of Figure 51.

FIGURE 55. *Schizoporella biaperta.* A moderately calcified zooecium showing two avicularia, the two apertural teeth (cardelles) and the sinus between them. A typical specimen.

FIGURE 56. *Schizoporella biaperta.* A heavily calcified zooecium topped by an ovicell. The zooecial shape is atypical and due to crowding in the colony and to excessive calcification. The avicularium is heavily calcified. The depressed rim of the ovicell frontal has been accidentally over-emphasized and should look less depressed (see Figure 54, middle ovicell). Drawn to same scale as Figure 55.

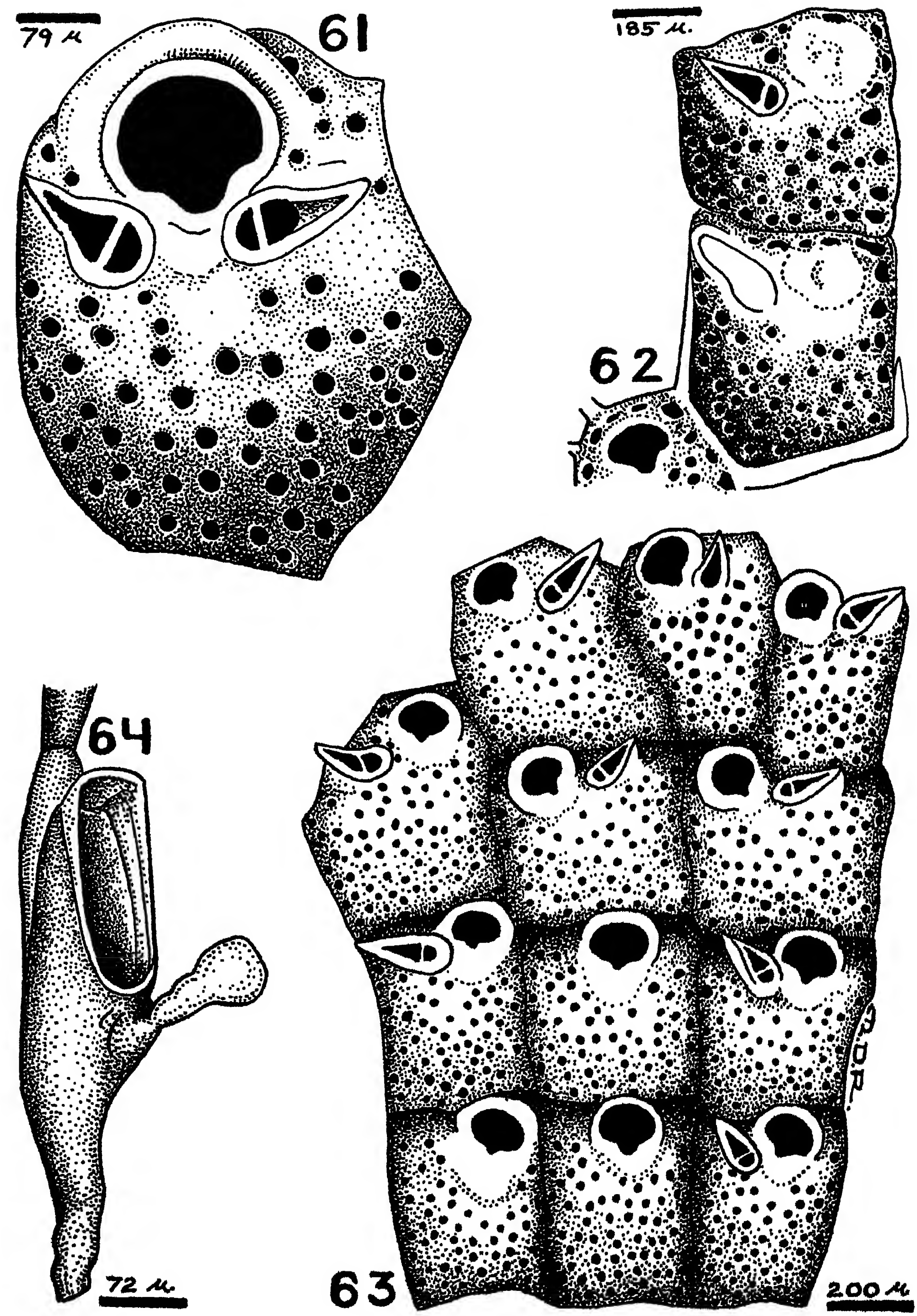
FIGURE 57. *Schizoporella unicornis.* A sharply pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 58. *Schizoporella unicornis.* Three zooecia, two of which have ovicells. The bottom zooecium is twice as broad as the other two to which it gives rise. The zooecial frontal wall and the ovicells have pores. The sharply pointed avicularia vary in size and are near the aperture.

FIGURE 59. *Schizoporella unicornis.* Another unusually shaped and very broad zooecium which would give rise to two rows of zoids. The pores of its frontal area and of the ovicell are better shown. Drawn to the same scale as Figure 54.

FIGURE 60. *Schizoporella unicornis.* A smaller pointed avicularium, drawn to the same scale as Figures 55 and 57.

PLATE IX



tion was not heavy. Some New Rochelle specimens had up to ten spines, while others had no spines around the aperture—all in the same colony. Some zoids had 11 tentacles.

This species was pictured in an earlier paper (Rogick, 1940, p. 167, Figs. 6–9).

Membranipora Tuberculata

A specimen of *Membranipora tuberculata* was found on fronds of Cryptopleura sp. and Sargassum sp. which were sent to the writers by Dr. William Randolph Taylor. The Cryptopleura had come from Puerto de la Paloma, Uruguay, from the collection of Carmen de Franco de Pimienta. The Sargassum sp. had been collected by Adrien Questel on April 21, 1944, from Guadeloupe, Antilles. This Membraniporan has been previously reported by Marcus from Laminaria (1939, p. 126) and Fucus (1937, p. 34); by Hastings (1929, p. 706) from Padina; and by Osburn (1912, p. 231) from *Sargassum bacciferum* which had drifted into Vineyard Sound.

The extensive colonies of *M. tuberculata* spread flatly over the algal fronds in an ivory-white lacework, reminiscent of *Electra pilosa.* The two or three prominent calcareous tubercles at the anterior end of each zooecium from which this species gets its name may project separately and distally or may coalesce, forming a somewhat rounded ledge.

Since *M. tuberculata* was adequately pictured in both Osburn's (1912, as *M. tehuelca*) and Marcus' (1937) papers, no figure of it was included in the present study.

Microporella Ciliata

(Figures 47–49)

Small, flat, circular colonies, white to irridescent in color, calcareous though fragile, encrust shells, rocks, and five algal species in the Woods Hole region. Prenant and Teissier (1924, p. 23) found *Microporella ciliata* on three additional algae: Himanthalia, *Laminaria saccharina* and *Saccorhiza bulbosa.* Hadley Harbor specimens were found growing on the same thallus with Foraminifera, *Aetea sica, Crisia eburnea, Hippothoa hyalina,* and *Schizoporella biaperta.*

Plate IX

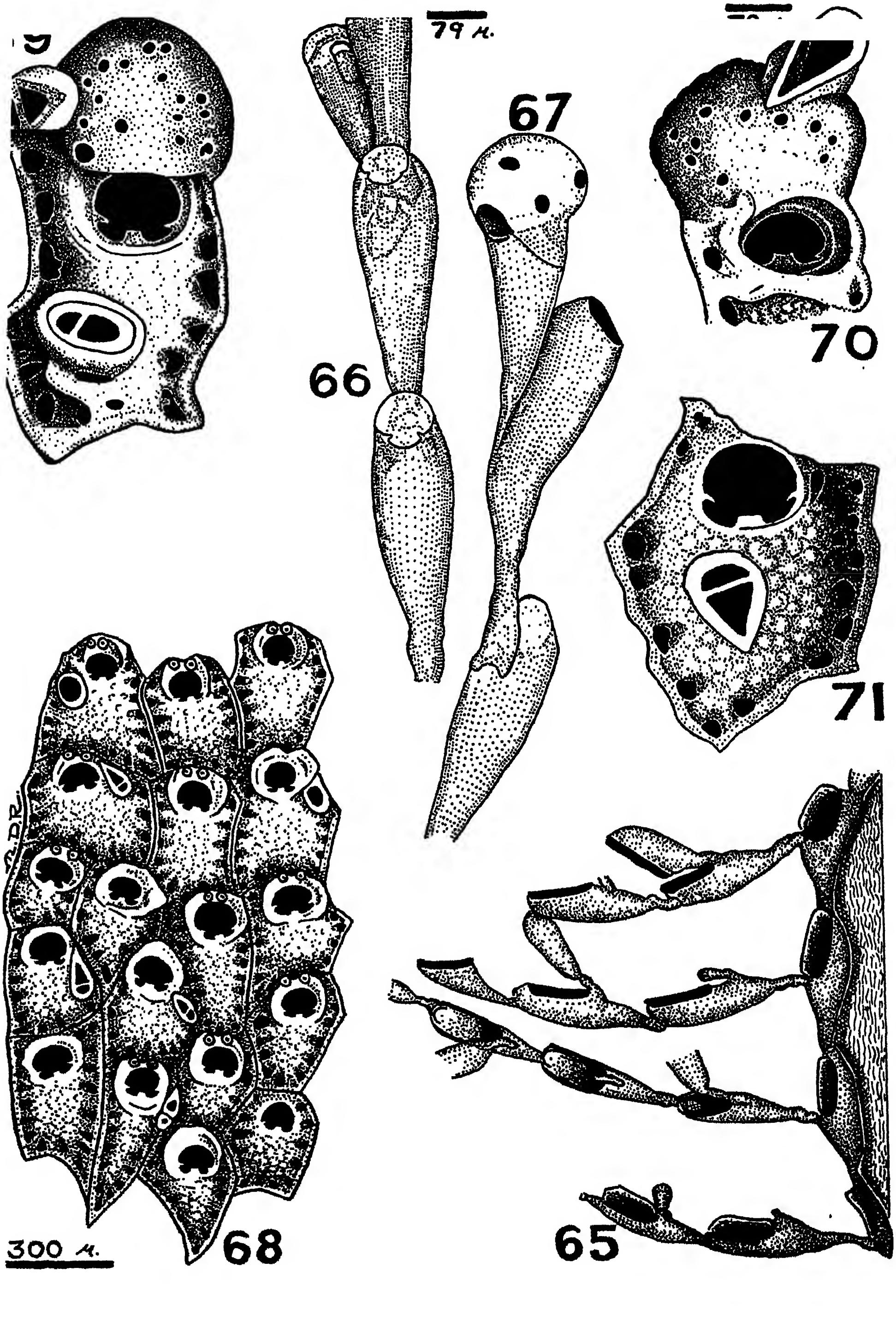
Figure 61. *Schizoporella unicornis.* A single zoid showing typical rounded aperture with its postral sinus and two pointed avicularia situated on either side of the aperture. The presence of two avicularia is a less frequent condition than the presence of one avicularium. Frontal surface of zooecium has a number of closely set pores. Calcined specimen.

Figure 62. *Schizoporella unicornis.* Three zooecia showing varying degrees of calcification. The uppermost square zooecium has a completely calcified aperture. The second squared zooecium has the aperture and avicularium openings completely calcified or plugged up. Around the right, left and lower sides of this second zoid are white septa, outgrowths from a newly overgrowing colony whose marginal zooecium is shown as the partial, third, bottom zooecium with black aperture.

Figure 63. *Schizoporella unicornis.* Portion of a typical colony. Three rows of zooecia are at the bottom and four at the top of this colony fragment, showing how a colony may increase in width at the periphery. Five zooecia have the avicularium on one side of the aperture, four on the other and three are without avicularia. Apertures may be placed either in the middle or at one side of the distal part of the frontal surface. The frontal surface is rather flat in this colony. Calcined specimen.

Figure 64. *Scruparia ambigua.* A zoid with a frontal and distal bud. The basal proximal part of the frontal bud and of the zoid is slightly twisted, a typical condition.

PLATE X



Some zooecia are without oral spines, avicularia or ovicells. Others have them. Oral spines may number from 3 to 7 (Figs. 47, 49). Ovicells are globose, "pebbled" in texture, and provided with small pores (Fig. 48). One avicularium is placed at an angle on either the right or the left frontal side, one-third to one-half of the way down and laterad from the aperture. The aperture is hemispherical and placed above the small crescent-shaped ascopore (Fig. 49). Canu and Bassler (1930, p. 47) reported 13 to 14 tentacles for this species.

Embryo-filled ovicells were collected on VIII–28–1939. Many very young colonies were found developing at that time also.

Pedicellina Cernua

(Figure 50)

Pedicellina cernua though small, soft-bodied, and inconspicuous was reported on 11 algal species. Also, Leidy (1855, p. 11) reported it on the "roots" of *Laminaria saccharina;* Prenant and Teissier (1924, p. 19) reported it on the Cystoseiras, Florideae and Dictyota; and Joliet (1877, p. 296) on *Corallina squammata* and *Cladophora rupestris.*

It has a creeping stolon from which arise flexible stalked zoids. Spines occurred on the calyx and stalk (Fig. 50) of a few zoids but most specimens were without them. The tentacle number in several very young zoids was 8 to 12. The number increases with age. Marcus (1939, p. 212) gives the tentacle range for this species as 8 to 24.

Plate X

Figure 65. *Scruparia ambigua.* A row of four zooecia encrusting a thick algal filament. From these arise four branches of zoids. The opesial rim of all but possibly the terminal budless individuals is parallel to the back of its own zoid. Drawn to the same scale as Figure 68.

Figure 66. *Scruparia clavata.* Two full-grown zooecia and two developing buds. The smaller, left bud belongs to a developing ovicell. The ovicelled individual originates below the other bud. Drawn to the scale above.

Figure 67. *Scruparia clavata.* Two grown zooecia and a third smaller one topped by a globose ovicell which has several large pores. The ovicell faces in the opposite direction from the ordinary zooecium. Drawn to same scale as Figure 66.

Figure 68. *Smittina trispinosa.* Eighteen ordinary zooecia growing in a very regular fashion. Some zooecia have rounded and some pointed avicularia while others have none at all. The flared peristome about the aperture is well developed in many. Some have spines, others do not. Calcined specimen.

Figure 69. *Smittina trispinosa.* A zooecium, ovicell and two avicularia. The circular aperture with the two cardelles (lateral teeth) and the lyrula (broader, median tooth) is a key character. So are the ovicell with its pores and leaning avicularium and the row of marginal zooecial areolae. Avicularia may be found in various locations on the frontal zooecial surface (compare this figure with Figures 68 and 71). The zooecium shows secondary calcification about the lower avicularium and partly covering the lower areolae. Drawn to same scale as Figure 70. Calcined specimen.

Figure 70. *Smittina trispinosa.* An ovicell topped by a triangular avicularium. The zooecial peristome is heavily calcified and forms a collar at the bottom of which can be seen the aperture, lyrula and cardelle.

Figure 71. *Smittina trispinosa.* A young zoid showing the characteristic apertural features and a frontal triangular or pointed avicularium. The frontal surface is slightly "beaded" and marginal areolae outline the thin edge. Drawn to same scale as Figure 70.

Schizoporella Biaperta

(Figures 51–56)

This white to reddish-orange bryozoan is fairly common on nine algal species. Its colonies attain a diameter of 2 cm. or more and appear fairly sturdy. They grow either flat on the thallus or may extend beyond the thallus, forming calcareous "ruffles" which may be lamellate (several layers in thickness).

The key characters of this species are: (1) rounded aperture with a postral sinus between the two cardelles (Fig. 55); (2) one or two small oval or ellipsoidal avicularia (Figs. 52, 53) mounted on mammillate prominences at the right or left or both sides of the sinus area (Figs. 51, 55); (3) frontal wall perforated by irregularly sized and spaced pores, and (4) hemispherical ovicells the edge of whose frontal area is slightly depressed and marked by faint calcareous ribs (Figs. 54, 56). Heavy calcification obscures some of these characters, especially the porous frontal area of the zooecium (Figs. 54, 56).

Twelve tentacles were counted on one zoid.

In July and August, the ovicells contained red embryos or larvae.

Schizoporella Unicornis

(Figures 57–63)

Schizoporella unicornis is very common on rocks and shells, but less frequent on algae. It grew on six Woods Hole region algal species. Additional algal hosts mentioned by Prenant and Teissier (1924, p. 23) are the Florideae, Himanthalia, and *Saccorhiza bulbosa.*

There is great variation in the appearance of the colonies. Their color ranges from white to reddish orange to a dull red. Some are smooth, flat, and shining, others rough and extended beyond the thalli. They may be lamellate, one colony growing over another. No description of the species is necessary because Figures 58 and 59 show the ovicells, and Figures 61 and 63 show the typical zooecial appearance, growth habit, aperture shape, and disposition of the avicularia. The avicularia grade in size (Figs. 57–60), but are always sharply pointed. Calcification may sometimes obliterate them and the aperture (Fig. 62).

In the Woods Hole area, larvae were found in ovicells in July and August (the times when collection was made) and undoubtedly occurred before and beyond these dates. At Beaufort, North Carolina, they are found the year round, according to McDougall. McDougall (1943, p. 340) observed that the times of greatest abundance of larvae (as judged by settlings on experimental substrata there at Beaufort) were in May, June, September, October and November.

Scruparia Ambigua

(Figures 64–65)

Most of the specimens of this dainty little dendritic form were dredged off Gay Head, Martha's Vineyard, on VII–30–1946. Some were growing on *Bugula turrita,* others on eleven algal species. It also grew in close association with hydroids and *Hippothoa hyalina.*

Scruparia ambigua zoids are yellowish, horny, transparent, and slender. They ranged from 0.345 to 0.495 mm. in length, the average of 12 specimens being 0.431 mm. The branching of the colony is quite open. Tentacles numbered ten in each of two zoids. No ovicells or larvae were found in the present material. Barrois (1877, p. 194) found ovicells and larvae at Roskoff during the month of June.

Hastings (1941) made a careful study of this species and differentiated *Scruparia ambigua* from *S. cheluta* on the basis of the opesial slant and encrusting zooecia. In *S. ambigua* the opesial rim is parallel to the basal wall of the zooecium, and the free zooecial branches arise from a series of encrusting zooecia, as in Figure 65.

Scruparia Clavata

(Figures 66–67)

A few scraps of this delicate, horny, dendritic, transparent bryozoan were growing on *Laminaria Agardhii* which was dredged off Gay Head, Martha's Vineyard on VII–30–1946. Some ovicells were present. The zooecia bearing them were slightly smaller (Fig. 67) than the other zooecia. The zooecial orifice is much smaller than that of *Scruparia ambigua*.

Marcus (1940, p. 208) created a new genus *Haplota* for *S. clavata*.

Smittina Trispinosa

(Figures 68–71)

This species was found with great frequency on shells and rocks, sometimes many layers in thickness on the latter. However, its occurrence on algae was infrequent. *Chondrus crispus* (from North Falmouth, Mass.), *Laminaria Agardhii*, *Phyllophora Brodiaei*, and *P. membranifolia* from Woods Hole had a few colonies.

Rock colonies or nodules often are a light mustard yellow color; colonies on algae, however, were never that striking a color, but were ivory or iridescent.

Colonies are very fine grained in general appearance. The species shows a great deal of variation, depending upon age, degree of calcification, and nature of the substratum.

Summary

1. A total of 30 bryozoan species was reported from 37 species of marine algae.

2. Five bryozoan species were reported from three species of green algae, 26 bryozoan species from 11 species of brown algae, and 27 bryozoan species from 23 species of red algae.

3. *Phyllophora membranifolia* yielded the greatest number of bryozoan species (23), *Chondrus crispus* and *Laminaria Agardhii* each yielded 20, *Phyllophora Brodiaei*, 15, and *Cystoclonium purpureum cirrhosum* yielded 11 bryozoan species.

4. Each of the three commonest bryozoa, *Aetea sica, Bowerbankia gracilis* and *Crisia eburnea,* was found on 18 algal species.

5. Each of the two next commonest bryozoa, *Electra pilosa* and *Hippothoa hyalina,* occurred on 17 red and brown algal species.

6. *Bugula turrita*, the next most common form, was found on 16 algal species.

7. *Crisia eburnea* and *Hippothoa hyalina* were common on over half the red algal species examined.

8. Some bryozoa seemed to grow most frequently and abundantly on certain algal species, namely:

a. *Alcyonidium polyoum* on *Chondrus crispus, Phyllophora Brodiaei,* and *P. membranifolia*

b. *Bowerbankia gracilis* on *Ascophyllum nodosum, Chondrus crispus, Fucus vesiculosus, F. vesiculosus spiralis, Phyllophora Brodiaei* and *P. membranifolia*

c. *Crisia eburnea* on *Chondrus crispus, Phyllophora Brodiaei* and *P. membranifolia*

d. *Electra pilosa* on *Laminaria Agardhii* and *Rhodymenia palmata*

e. *Flustrella hispida* on *Ascophyllum nodosum.*

9. To the 84 known Woods Hole region bryozoan species can be added three more: *Aetea sica, Cellepora dichotoma* and *Scruparia ambigua.* It is quite possible that some of the previously reported *Aetea anguina* and *Cellepora americana* material may have included *Aetea sica* and *Cellepora dichotoma,* respectively.

10. Algal collections from New Rochelle, N. Y., yielded some of the same bryozoan species as are found in Woods Hole, namely: *Alcyonidium polyoum, Bowerbankia imbricata, Cryptosula pallasiana, Electra hastingsae, Membranipora lacroixii* (?), and *Pedicellina cernua.*

11. Algal collections from Rye, N. H., yielded some of the same bryozoa as are found at Woods Hole, namely: *Callopora aurita, Cribrilina annulata, Cribrilina punctata, Crisia eburnea, Electra pilosa, Hippothoa hyalina* and also a form, *Lichenopora hispida,* which did not occur at Woods Hole.

12. Twenty-six of the thirty bryozoan species were carefully illustrated.

13. Tentacle number counts were made for 15 species.

14. Three bryozoan species were collected, observed, or known to be in the larva-producing stage on algae in late June; seven species in July; ten in August; two in September. These were chance observations and the number of species would have been greater if more exhaustive collections over a greater number of months could have been made.

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STRATIFICATION AND DEFORMATION OF ARBACIA PUNCTULATA EGGS CENTRIFUGED IN CAFFEINE SOLUTIONS

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Introduction

During an investigation of the effect of methylated purines upon cellular behavior, the following problems arose: Does contact with the trimethylated purine, caffeine, which is known to influence cellular metabolism, affect the viscosity of the cytoplasm of the cell; and is there any evidence that this alkaloid influences the forces at the cell surfaces? Heilbrunn's informative studies (1926, 1928, 1943) on viscosity and surface forces, with reference to numerous chemical substances, did not deal adequately either with alkaloids as a group, or with caffeine in particular. Centrifugation followed by a study of the degree of granule stratification within the cytoplasm, together with changes in form of the cell itself, offered a satisfactory method of approach to these questions. From the experiments described below, it will be seen that caffeine does not alter the viscosity of the unfertilized egg but acts upon the membrane and cortical tension forces, thereby influencing cleavage in the fertilized egg. In the higher concentrations, even sperm entry is prevented by caffeine.

The temperature factor is not significant in the work reported here, since caffeinized eggs were centrifuged at the same time and temperature as control eggs from the same female. In this way, relative viscosity effects could be observed.

Methods and Materials

The author (1945, 1946a, b, 1946) has demonstrated by studies on O_2 consumption and comparative sensitivity of developmental stages that caffeine retards cleavage in Arbacia. In the present study, the unfertilized and fertilized eggs [unfertilized in sea water (SW), unfertilized in 0.10 per cent caffeine-in-sea-water (CSW); fertilized, i.e. normal egg (N ♀) × normal sperm (N ♂), in sea water, and fertilized N ♀ × N ♂ in 0.10 per cent caffeine-in-sea-water] were centrifuged at 10,000 × g for five, seven, and twelve minutes, and also at 3000 × g, 40 minutes after fertilization or at the equivalent time interval after shedding in the case of unfertilized eggs. This 40 minute period was chosen because that is when the viscosity of the protoplasm approaches the increased state typical at the time of cleavage. Comparable series employing other concentrations were also run.

Experiments were conducted at the temperature of running sea water. The appearance of uncentrifuged and centrifuged caffeinized eggs was compared with photomicrographs and descriptions by E. B. Harvey (1940). Differences in the degree of stratification (compactness) of the pigment granules and vacuoles and the height of the hyaloplasm zone after centrifugation were noted as evidence of

relative viscosities. To avoid error due to a time variable caused by the return of granules by Brownian movement, all photomicrographic records were made ten minutes after centrifugation.

Results and Discussion

Cleavage abnormalities in eggs centrifuged in CSW were no greater than those observed in the same concentration of CSW without centrifugation. There was no evidence that caffeine induced any primary change in viscosity which would prevent cleavage. Clearly defined effects were reproducible and similar in both unfertilized and fertilized eggs, but those in the former were more convincing because the normal viscosity changes during mitosis made it impossible to assume that controls and experimentals would be in exactly the same state.

Bank (1932), using *Arbacia punctulata*, reported stratification within the unfertilized eggs without centrifugation if they were held in 1 per cent caffeine for 48 hours. This is not surprising since caffeine was shown by the author (1945, 1948) to retard the O_2 uptake of the Arbacia cell. The facility with which the egg contents stratify due to such a factor as O_2 uptake cannot be determined by centrifugation. In the experiments described in this paper, an indication of a surface effect was the fact that Arbacia eggs cannot be fertilized when immersed in 1 per cent CSW. Both the eggs and sperm, however, survive for a considerable period in 1 per cent CSW, and the eggs can be fertilized and undergo partial development if transferred to sea water. Therefore, this concentration of caffeine does not destroy the internal physiological potentialities of these gametes with respect to fertilization. Over as long a period as 48 hours, the physical effect noted by Bank can be understood on the basis of the biochemical inhibition of cellular respiration, and/or as a surface effect, without assuming a primary viscosity change due directly to caffeine.

Among the results observed in the present series, the delay of deformation, reduction in actual fragmentation, and the sharper margins of the layers (apparent under the earlier conditions of the experiments) were the most readily distinguishable and clearly associated phenomena. De Vries (1947), in his studies on viscosity and tension at the surface in eggs of the fresh water snail, *Limnaea stagnalis* L., based the interpretation of his results primarily on the occurrence of vacuoles and granules in the hyaloplasm zone, as well as on the height of this zone. He pointed out that the height of the hyaloplasm zone depends on both viscosity and the degree of stretching, i.e. tension at the surface. Therefore, it occurred to the present writer that the closer packing of the pigment granules might be attributable to the fact that the pigment in the spherical cell had a shorter distance to fall than the pigment in the uncaffeinized normal cell, which is always elongated by centrifugal force to the degree applied in the experiments up to this time. It seemed desirable to eliminate the effect of the stretching factor and resulting deformation in order to clarify the significance of the degree of stratification in interpreting viscosity changes. Accordingly, unfertilized and fertilized eggs, both control and experimental, were centrifuged at only 3000 × g for intervals varying from one-half to three minutes. These shorter centrifugations at 3000 × g did not change the shape of the cells in either the control or the

caffeinized eggs, but did allow stratification. Therefore, comparisons of stratification could be made without the deformation factor.

In the absence of internal viscosity changes, the increased force required to break caffeinized eggs indicates a surface effect. Harvey (1931) estimated that the centrifugal force necessary to pull the Arbacia egg into two halves indicates that tension at the surface for a 25 per cent increase in area is less than 0.2 dyne per cm. with considerable variation in eggs. At 10,000 × g for 12 minutes, a count of five fields of each of the experimental caffeine series showed the percentage of breaking in the unfertilized eggs to be as follows: Controls in SW were 100 per cent broken; eggs in 0.02 per cent CSW, 12 per cent broken; in 0.10 per cent CSW, 0.50 per cent were broken; and in the 2.0 per cent CSW, only 0.08 per cent were broken although slight elongation did occur.

The apparent absence of any significant osmotic change (Cheney, 1948) as well as of demonstrable viscosity changes in the internal protoplasm, together with the delay in deformation reported here, would indicate that caffeine may initiate a change in the surface of the cell. Such an effect might involve both the membrane and the cortical protoplasm, which Harvey and Shapiro (1941) demonstrated to possess a considerably higher viscosity than the interior protoplasm in the eggs of *Arbacia punctulata* and *Asterias forbesii.*

Summary *

1. Caffeine does not change the existing viscosity state of the egg.

2. Egg fragmentation, under centrifugation, decreases with increased caffeine concentration.

3. The "apparent" effect of greater stratification of the granules in Arbacia eggs centrifuged in caffeine does not occur if the centrifugal force to which the eggs are subjected is sufficient to produce sedimentation but insufficient to cause deformation.

4. Evidence indicates that the delay of deformation in the caffeinized eggs, centrifuged at 10,000 × g or less, may be due to the action of caffeine (trimethylated purine) upon the total tension forces at the surface areas of both unfertilized and fertilized Arbacia eggs.

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A MUCIN CLOT REACTION WITH SEA-URCHIN FERTILIZIN

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Introduction

Recent work on the fertilizins (the sperm-agglutinating constituents of egg water) of the eggs of sea-urchins and other animals has shown them to be of the nature of mucoproteins. Tyler and Fox (1939, 1940) showed that the fertilizins of Strongylocentrotus and of Megathura possess protein characteristics, but are of low nitrogen content. Similar evidence has been obtained with Arbacia fertilizin by Kuhn and Wallenfels (1940) and with Psammechinus fertilizin by Runnström, Tiselius, and Vasseur (1942). The latter workers also obtained a positive carbohydrate test. Tyler (1948) reported the presence of reducing sugars to the extent of about 15 per cent in hydrolyzed, purified preparations of Strongylocentrotus fertilizin and identified galactose as one of the constituents. As will be shown later in this paper, the present author has found hexosamine to be present in amounts equivalent to about 2 per cent of the original material. According to Runnström, Tiselius, and Vasseur (1942) and Tyler (1946) the sea-urchin fertilizins are of pronounced acidic character.

Acidic mucopolysaccharides are known (cf. Meyer, 1945, and Stacey, 1946) to co-precipitate with proteins upon acidification of the native fluid or of the neutral extracts in the form of "mucin clots," stringy or granular precipitates, depending upon the conditions of precipitation. This reaction is given, for example, by hyaluronic acid (Meyer and Palmer, 1936) and has been used in the assay of the enzyme hyaluronidase (McClean, 1943). It was of interest to determine whether or not fertilizin preparations would give the mucin clot reaction. As the work reported here shows, fertilizin preparations do give such a mucin clot reaction. A titration method, based upon this, was developed for these preparations, and comparisons made with their sperm-agglutinating activity in untreated condition, after dialysis, and after exposure to heat and to ultra-violet irradiation.

Materials and Methods

The sea-urchins *Lytechinus pictus, Strongylocentrotus purpuratus* and *S. franciscanus* were used in these experiments. Most of the work was done with *S. purpuratus.*

Two kinds of fertilizin preparations were employed. One, which will be termed "crude fertilizin," was prepared by acidifying a 20 per cent suspension of washed eggs to pH 3–3.5, removing the supernatant fluid after five or ten minutes and readjusting the pH of this solution to 7–7.5. The other, which will be termed "purified fertilizin," was further subjected to alkali precipitation, dialysis against 3.3 per cent acid saline (pH 3.5–4) and alcohol precipitation according to the method described by Tyler (1948). Material prepared in this manner has been

found to be electrophoretically homogeneous (Tyler, unpub.). For the various tests the solutions were made up in 3.3 per cent NaCl at a pH of about 7.

Sperm-agglutinating titer of the fertilizin preparations was determined using the drop method of preparing two-fold serial dilutions with sea water in Syracuse watch glasses. To two drops of each dilution of fertilizin solution one drop of a uniform sperm suspension, usually 1 per cent (calculated as 1 cc. dry sperm per 100 cc. sea water suspension), was added. The highest dilution in which agglutination is observable under the microscope gives the titer of the preparation.

Tests for univalent fertilizin were made according to the method described by Tyler (1941) and Metz (1942). Essentially, this method consists of first treating sperm with the solution containing univalent fertilizin, which does not agglutinate the sperm, and then adding an equal volume of strong normal fertilizin solution to the suspension of sperm. Failure of the sperm to be agglutinated by the normal fertilizin presumably indicates that the combining groups on the sperm surface have been occupied by univalent fertilizin groups and are no longer available to unite with the normal fertilizin. Univalent fertilizin was obtained by heating and by irradiation with ultra-violet light of normal fertilizin preparations (cf. Tyler, 1941, and Metz, 1942).

Bovine serum albumin prepared by the Armour laboratories was used in 1 per cent solution for the co-precipitation tests.

Hyaluronic acid was obtained from human umbilical cords according to the method described by McClean (1943), whereby the distilled water extract of acetone-dried, ground cords, extracted with 90 per cent acetic acid according to the method of Meyer and Palmer (1936), was precipitated with 1.25 volumes of cold, potassium acetate-saturated 95 per cent alcohol. The precipitate was washed with alcohol, acetone, and ether and dried over P_2O_5. The dry product was dissolved in distilled water as required; a solution of 0.1–0.2 per cent of the dry material was clear, viscous and did not form a precipitate upon the addition of acetic acid, but co-precipitated with serum albumin in the presence of acetic acid, forming a stringy clot. In higher dilutions the mixture of serum albumin, acetic acid and hyaluronic acid solution resulted in the formation of a fine precipitate or turbidity. The highest dilution in two-fold serial dilutions in which turbidity was perceptible by visual inspection was taken as the titer of the hyaluronic acid solution.[1]

Experiments and Observations

Co-precipitation of fertilizin with serum albumin in acid solution

A viscous solution of crude fertilizin of *Lytechinus pictus* was prepared as described above and combined with a 1 per cent solution of bovine serum albumin in 0.9 per cent NaCl and 2 N acetic acid according to the method described by McClean (1943) in the mucin clot test. With this solution a very large clot was formed similar in character and appearance to the clot formed by hyaluronic acid. Purified fertilizin preparations of *Strongylocentrotus purpuratus*, *S. franciscanus*, and *L. pictus* were tested in the same manner and in each case a clot or precipi-

[1] A quantity of pure potassium hyaluronate was later supplied to me by the Schering Corporation, through the courtesy of Dr. W. Alan Wright and Dr. Erwin Schwenk.

tate formed, depending upon the concentration of the solution. In Figure 1 a series of photographs of the mucin clot reaction of *S. purpuratus* fertilizin is shown, with the reaction given by hyaluronic acid for comparison.

By taking advantage of the biological activity of fertilizin, i.e., its sperm-agglutinating activity, a simple test was performed which provided definitive evidence that it is the fertilizin which is co-precipitated with serum albumin and not some hitherto undetected component of the material.

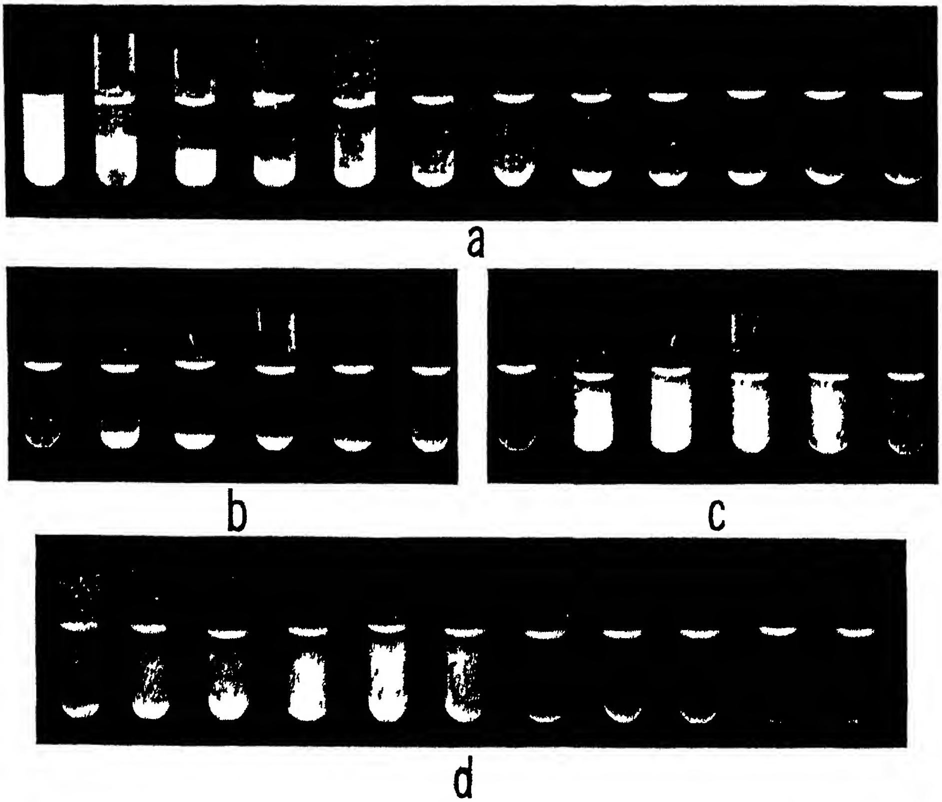

FIGURE 1. Mucin clot reactions of *Strongylocentrotus purpuratus* fertilizin and of hyaluronic acid in two-fold dilutions. a. Purified *S. purpuratus* fertilizin. The first tube contains fertilizin solution and serum albumin, but distilled water instead of acetic acid. The opacity of the first tube is due to the opalescence of the mixture. b. Crude *S. purpuratus* fertilizin. The first tube contains fertilizin solution and acetic acid but 0.9 per cent saline instead of serum albumin. c. The same as b with the tubes shaken prior to being photographed. d. Hyaluronic acid.

A solution of purified fertilizin in 3.3 per cent saline was mixed with serum albumin and the pH brought to about 3.5 with 2 N acetic acid. The resulting precipitate was thrown down by centrifugation, the supernatant withdrawn and its pH adjusted to 7. As shown in Table I, the supernatant exhibited no sperm-agglutinating activity.

TABLE I

Sperm-agglutinating activity of the supernatant and of the precipitate recovered separately after the addition of bovine serum albumin to an acidified solution of purified fertilizin, and results of control tests

Reaction mixture		Per cent of sperm-agglutinating activity*
0.5 ml. fertilizin in 3.3% saline	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	Supernatant: 0 Dissolved precipitate: 100
	0.05 ml. distilled H_2O 0.2 ml. 1% bovine serum albumin in 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 0.9% saline	100
	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline, acidified and neutralized	100
0.5 ml. 3.3% saline	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	0

* Sperm-agglutinating activity of fertilizin, distilled water and 0.9% saline mixture taken as 100%.

It had previously been found that the precipitate formed by the addition of serum albumin to a fertilizin solution acidified to a pH of about 3.5 dissolves completely at a pH of 5.6 or higher. After centrifugation and withdrawal of the supernatant of the material being tested, the precipitate was resuspended in 3.3 per cent NaCl solution, its pH brought to about 7, and the volume made equal to that of the original mixture. Upon testing this solution it was found, as is shown in the table, that the sperm-agglutinating activity was equal to that of the original fertilizin solution, showing that the activity was recovered quantitatively from the precipitate.

A number of control tests were made, the results of which are summarized in Table I. These showed that (1) co-precipitation of fertilizin and serum albumin does not occur in the absence of acid; (2) it does not occur in acidified solution in the absence of added protein; (3) the presence of albumin does not affect the sperm-agglutinating activity of the fertilizin; (4) the sperm-agglutinating activity is not affected by acidification and subsequent neutralization of the solution, and (5) sperm agglutination does not occur in saline solution in the absence of fertilizin, nor is a precipitate formed when albumin and acid are added to saline.

Quantitative recovery of the sperm-agglutinating activity from co-precipitated fertilizin and serum albumin, which is achieved by the simple expedient of raising the pH to 5.6 or higher, shows that the specific combining groups of the fertilizin are not irreversibly altered by its reaction with the albumin. The significance of this phenomenon cannot be evaluated at the present time, however, since essentially nothing is as yet known of the structure of the fertilizin molecule.

Titration by the mucin clot method

The method employed for titration was as follows: Two-fold serial dilutions of fertilizin in 3.3 per cent NaCl at about pH 7 are made in 10 × 75 mm. test tubes in 0.5 ml. quantities. To each tube 0.05 ml. of 2 N acetic acid is added and the contents mixed. The tubes are inclined and 0.2 ml. of 1 per cent bovine serum albumin in 0.9 per cent saline is slowly pipetted down the side of each tube; after this the tubes are carefully returned to a vertical position and in most cases a ring of precipitate forms immediately at the zone of contact between the albumin solution and the acidified fertilizin solution. The rings are easily detected at high dilutions in which a diffuse turbidity is difficult to detect and score visually. The reactions are read at once in good natural light, since the rings tend to disperse rapidly as the albumin diffuses through the mixture. The highest dilution at which a ring is observed is taken as the mucin clot titer of the preparation.

Effect of pH, albumin concentration and salt concentration

Numerous titrations with the same stock samples of purified fertilizin using the method described above have given consistently identical mucin clot titers in the course of routine testing. It was thought advisable, however, to carry out some controlled tests to determine in a more definitive manner the amount of variability in titer to be expected within a rather limited range of pH, albumin concentration, and salt concentration.

A sample of a purified fertilizin solution from eggs of *S. purpuratus* was centrifuged at 3000 r.p.m. for five minutes. A slight sediment was thrown down, the clarified supernatant was drawn off, and the pH adjusted to 7.0 with the glass electrode. To assure maximum uniformity of different samples of the supernatant, it was thoroughly mixed before removing an aliquot. Two-fold dilutions were made in 0.5 ml. quantities with 3.3 per cent NaCl solution. Serum albumin was made up in 1 per cent solutions, and the same stock solution of 2 N acetic acid was used throughout. Various amounts of the latter two solutions were added to different sets of tubes of the fertilizin dilutions. The total albumin and salt concentration in the different sets was thus altered, as noted below. The pH was measured with a Beckman glass electrode pH meter. After each titration the pH of the final mixtures was measured in the first tube, in the tube giving the end-point, and in an intermediate tube. The maximum difference in pH observed within any set was 0.26 unit. The results obtained in a series of nine titrations are given in Table II. Each of the pH values listed represents the mean of three determinations in each set. The total salt concentration is expressed as per cent NaCl. The mucin clot titers (last column of table) are the end points of visible precipitation as determined by the ring method described above.

As the data in the table show, no marked difference in mucin clot titer occurs, for the most part, as a result of the differences in pH, total salt and albumin concentrations employed. In titration 5, the observable end-point would probably have been at least one dilution higher had rings formed. The failure of rings to form is attributable to the relatively large quantity of serum albumin solution used; as noted above, in high dilutions a diffuse turbidity such as was produced in this case is more difficult to detect visually than a ring at the same dilution. It has

been found that 0.5 ml. of albumin solution is about the largest quantity practicable for obtaining consistent ring formation. Since there is always a tendency to pipette too rapidly when a large number of tests is being performed, it has proven more convenient to use 0.2 ml. of albumin solution.

The evidence presented in Table II indicates that pH is not a critical factor with respect to observed titer within the range tested. The albumin concentration is not critical, nor is the total salt concentration of the system in the range from 2.0 per cent to 3.1 per cent. Below 2.0 per cent the salt concentration may be a more important factor, as shown by the higher titer obtained in titration 6. Although the total salt concentration in titration 5 is the same as that in number 6 (1.6 per cent), the two sets are not comparable for the reason mentioned above.

TABLE II

Mean pH; total albumin concentration, total salt concentration and mucin clot titers in nine titrations using uniform samples of a homogeneous purified fertilizin preparation of S. purpuratus

Titration no.	Ml. 2 N acetic acid	Ml. 1% albumin solution	NaCl concentration of albumin solution, per cent	Mean pH	Total albumin concentration, per cent	Total NaCl concentration, per cent	Mucin clot titer
1	0.05	0.1	0.9	3.09	0.15	2.7	64
2	0.05	0.2	0.9	3.30	0.27	2.4	64
3	0.05	0.3	0.9	3.33	0.35	2.3	64
4	0.05	0.5	0.9	3.54	0.47	2.0	64
5	0.05	1.0	0.9	3.74	0.65	1.6	32*
6	0.05	0.5	0.0	3.58	0.47	1.6	128
7	0.05	0.5	3.3	3.58	0.47	3.1	64
8	0.01	0.2	0.9	3.88	0.28	2.6	64
9	0.02	0.2	0.9	3.78	0.28	2.5	64

* Scored as turbidity, not as ring.

In the next section it will be shown that the mucin clot and sperm-agglutinating titers of a fertilizin solution are both reduced after dialysis of the preparation against distilled water with consequent removal of salt (NaCl). The data to be presented indicate that the physical state of the fertilizin is reversibly modified in the absence of electrolytes, at least under certain conditions of temperature. It seems likely, therefore, that there may exist some optimum concentration of electrolytes between zero concentration and that represented by 2.0 per cent NaCl (equivalent to an ionic strength of 0.34) at which co-precipitation of fertilizin and serum albumin attains a maximum. Further analysis of the effect of salt concentration on the co-precipitation of fertilizin and serum albumin is under way and will be reported in a later communication.

In the system as employed in the routine titration procedure in the present investigation, however, the mean pH, total albumin and total salt concentration fall well within the range for each of these factors in which identical titers are obtained, using uniform samples of a homogeneous fertilizin preparation.

Mucin clot and sperm-agglutinating titrations of fertilizin in salt-free solution

A sample (I) of a stock preparation of purified fertilizin in 3.3 per cent saline was made salt-free by dialyzing against distilled water at 1° C. until it no longer formed a precipitate with $AgNO_3$. It was found that a white, flocculent material was present in the dialyzed solution, whereas no such flocculence was evident in the control in 3.3 per cent NaCl solution which was kept at 1° for the same period. A considerable reduction in both mucin clot and sperm-agglutinating titer of the salt-free sample as compared with the control was observed. In the mucin clot titration, the character of the precipitate formed in the salt-free preparation upon the addition of serum albumin in the presence of acid differed from that in the control in being of larger particle size and somewhat stringy. Difficulty was encountered in making sperm-agglutinating titrations (where the salt content of the preparation was adjusted just prior to titration to 3.3 per cent NaCl by adding an equal volume either of 6.6 per cent NaCl or of double sea water to the salt-free solution) in cases where suspended material was present. The spermatozoa clumped about the particulate matter, and it was impossible to score the dilutions satisfactorily. Where tests were made with samples of the dialyzed solution in which the amount of suspended material was visibly less than was originally present, both the mucin clot precipitation and sperm agglutination occurred in the manner characteristic of the control solutions; in these cases the two titers were also somewhat higher, although they did not necessarily equal the values obtained for the control solution.

A second sample (II) of the same stock preparation of purified fertilizin was dialyzed against distilled water until salt-free. This time the first five changes of the water used for dialysis, totaling 3 liters, were saved, combined, and lyophilized to dryness. The residue was taken up in about 10 cc. of distilled water and the resulting solution was approximately isotonic with sea water, as shown by the fact that sperm of *S. purpuratus* remained active when placed in it. This "dialysate-concentrate" was 300 times more concentrated than the original dialysate, but proved to be negative for both mucin clot formation and sperm-agglutinating activity. Sample II behaved in all respects like sample I. The results of tests with the two samples are summarized in Table III.

The evidence obtained from the present experiments indicates that at temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes. Macroscopic aggregates may appear in a fertilizin preparation under these conditions, and there is a correlated decrease in the mucin clot and sperm-agglutinating titers. Under the influence of added salt and elevated temperature (up to 21.5° C.), either separately or combined, there occurs a correlated decrease in the amount of visible macroscopic material and increase in mucin clot and sperm-agglutinating titers to values approaching those obtained with control solutions. The negative results of tests with the "dialysate-concentrate" show that there was no actual loss of fertilizin during dialysis.

Analysis of a purified fertilizin preparation

The co-precipitation of fertilizin with protein in acidic solution in a manner analogous to the behavior of acid mucopolysaccharides suggests affinity of fertilizin with this class of substances. It has recently been claimed, moreover, that a preparation

TABLE III

Results of titrations of salt-free fertilizin preparations of S. purpuratus after various treatments

Test no.	Fertilizin preparation	Treatment after removal from dialysis bath at 1° C.	Sperm-agglutinating titration		Mucin clot titration	
			pH	Titer	pH	Titer
1	I	None			5.2	8
2	I	Salt content adjusted to 3.3% NaCl. pH adjusted	7.2	64		
3	I	Salt content adjusted to 3.3% NaCl			5.2	16
4	I	2.5 hrs. at 21.5° C.			5.2	16
5	I	2.5 hrs. at 21.5° C. pH adjusted			7.2	16
6	I	Dialyzed vs. 3.3% NaCl at 1° C. 2 hrs. at 21.5° C. pH adjusted	7.0	64	7.0	16
7	I	Dialyzed vs. 3.3% NaCl at 1° C. 24 hrs. at 8° C. pH adjusted	7.45	1024	7.45	32
8	I	Control. Not dialyzed vs. distilled water. pH adjusted	7.4	512	7.4	64
9	II	1 hr. at 21.5° C. pH adjusted			6.9	32
10	II	Salt content adjusted to 3.3% NaCl. 3 hrs. at 21.5° C. pH adjusted	6.9	512		
11	II	Salt content adjusted to 3.3% NaCl. 1 hr. at 21.5° C. pH adjusted			6.9	64
12	II	Control. Not dialyzed against distilled water. pH adjusted	7.3	1024	7.3	128

from bull testes, presumably containing the enzyme hyaluronidase, is capable of causing the jelly of intact sea-urchin eggs to swell (Ruffo and Monroy, 1946; Monroy and Ruffo, 1947). It was of interest, then, to attempt to determine the extent to which fertilizin may be chemically similar to hyaluronic acid. The few available data, which have been reviewed briefly in the introductory section, indicate that fertilizin is by no means identical with hyaluronic acid. The present investigation has shown that fertilizin differs from hyaluronic acid to a marked degree with respect to the two chief constituents of the latter substance, hexosamine and glucuronic acid.

A homogeneous sample of a purified fertilizin preparation in 3.3 per cent NaCl was prepared as described above. In the present instance the supernatant fluid was filtered through hardened filter paper. The pH of the filtrate was adjusted to 7 and

TABLE IV

Results of chemical analysis of a purified fertilizin preparation of S. purpuratus

Filtrate					Hydrolysate	
Sperm-agglutinating titer	Mucin clot titer	Dry weight, mg./ml.	Total nitrogen, per cent	Glucuronic acid	Hexosamine, per cent	α-amino acids
2048	512	8.95	4.1	none	1.6	positive Ninhydrin

mucin clot and sperm-agglutinating titers were obtained. Aliquots of the filtrate were taken for the various analyses, which included determinations of dry weight, total nitrogen, hexosamine, α-amino acids and glucuronic acid. The results of the analyses are presented in Table IV. Dry weight per ml. was calculated from the weight of material precipitated from an aliquot of the filtrate with 1.25 volumes of cold 95 per cent alcohol. According to Tyler (1948), precipitation of fertilizin is complete under these conditions. The precipitate was washed with alcohol and dried in an oven at 55° C. to constant weight. Total nitrogen was determined for duplicate samples of the dried precipitate by the micro-Kjeldahl method. Another portion of the dried material was hydrolyzed by boiling in a sealed tube with 4N HCl for eight hours. Hexosamine was determined in an aliquot of the hydrolysate by the method of Palmer, Smyth and Meyer (1937). Another portion of the hydrolysate was treated with Ninhydrin reagent for the determination of α-amino acids. For the determination of glucuronic acid the colorimetric method recently described by Dische (1947b) was employed, using a sample of the original filtrate,

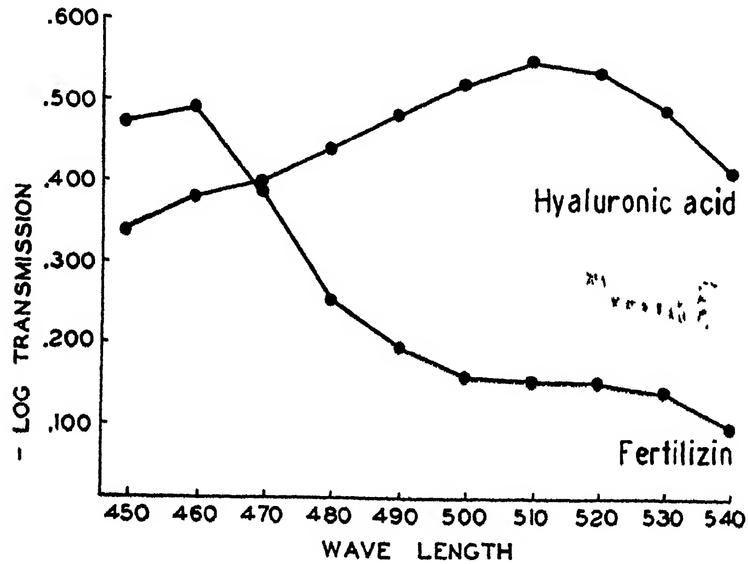

FIGURE 2. Absorption curves of reaction mixtures of mannose-thioglycolic acid-fertilizin and mannose-thioglycolic acid-hyaluronic acid. Wave lengths in mμ. Fertilizin $\Delta E_{(510-480)} = -0.097$; hyaluronic acid $\Delta E_{(510-480)} = +0.115$.

and for comparative purposes, a solution of pure potassium hyaluronate (Schering) was tested at the same time by the same method. According to Dische, the reaction of carbohydrates with -SH compounds in H_2SO_4, which differentiates between various classes of carbohydrates and individual hexoses and hexuronic acids, is highly characteristic for glucuronic acid when mannose is employed. This reaction is the basis of the test. The reaction mixture with glucuronic acid gives a typical absorption curve in the range 450–540 mμ, and it was found by Dische that the curve for hyaluronic acid is almost identical with that of glucuronic acid. In practice, accord-

ing to Dische, it is only necessary to measure the intensity of the mannose reaction at 510 and 480 mμ and subtract the second value from the first. This difference is positive for glucuronic acid and polyglucuronides, and negative for the other hexuronic acids. Figure 2 shows the absorption curves for fertilizin and for hyaluronic acid. The difference between the intensity of the mannose reaction with fertilizin at 510 and that at 480 mμ is negative, and hence it may be concluded that fertilizin does not contain glucuronic acid. This result is in agreement with previous results obtained by Tyler (unpub.) using an earlier method of Dische's (1947a). As shown in Table IV, fertilizin does contain hexosamine, but in small amount, which is in agreement with earlier results obtained by the present author using Kunitz's (1939) method. The Ninhydrin reaction was very weak but probably positive. Total nitrogen (4.1 per cent) of this material is somewhat lower than has been reported previously for *S. purpuratus* fertilizin by Tyler and Fox (1940), who found an average total nitrogen content of 5.2 per cent with crude preparations.

These data show that fertilizin differs markedly from hyaluronic acid in its chemical constitution. It is obvious, therefore, that the ability to give the mucin clot reaction does not by any means indicate close similarity between fertilizin and hyaluronic acid, even though it may be evidence that the former is related to the group of acidic mucopolysaccharides.

At the suggestion of Dr. Albert Tyler, the mucin clot titration procedure was used in conjunction with sperm-agglutinating titrations to investigate the effects of various kinds of treatment on fertilizin. In the following sections the results of parallel titrations of preparations subjected to heat and to ultra-violet irradiation are presented.

Parallel titrations with heat-treated fertilizin solutions

According to Tyler and Fox (1940), the sperm-agglutinating activity of *Strongylocentrotus purpuratus* fertilizin is rapidly destroyed at 100° C. The rate of inactivation, according to these authors, is a function of the pH, the fertilizin being most stable in the range from 4 to 7. Their data show that at pH 7.3 the agglutinin is 95–100 per cent inactivated in 20–30 minutes at 100°. In the present experiments in which *S. purpuratus* purified fertilizin solutions were used, the preparations have proven to be considerably more heat-stable than the material used by Tyler and Fox. Since the solutions employed by Tyler and Fox corresponded to crude fertilizin as defined in this paper, it may well be that the relatively purer condition of the fertilizin in the present preparations accounts for its greater stability.

Initial loss of agglutinating activity does not appear to involve complete destruction of fertilizin. At first the agglutinating fertilizin is converted into a "univalent," non-agglutinating form (cf. Tyler, 1941, Metz, 1942). It was of interest, accordingly, to test samples of heat-treated fertilizin for their univalence (inhibition) titer as well as for their sperm-agglutinating and mucin clot titers. The method employed for detecting univalent fertilizin has been briefly described in an earlier section of this paper; determination of inhibition titer consists in determining the greatest dilution in which no agglutination occurs upon the addition to the test dilutions of equal amounts of normal (untreated) fertilizin solution (Metz, 1942).

In the first experiments 1.5 ml. samples of the stock purified fertilizin solutions were placed in 13 × 100 mm. test tubes and immersed in a boiling water bath. The

exposed portions of the tubes were cooled by means of a stream of air so that heating could be continued for long periods without appreciable loss of fluid. Since sperm are quickly inactivated in even slightly hypertonic medium, the fact that the sperm remained active in the solutions that had been heated was assumed to indicate that evaporation of water from the tubes during heating was insignificant. A thermometer placed in the water bath with the tubes showed that the temperature of the bath fluctuated between 96° and 98° C.

In later experiments a quantity of fertilizin solution large enough to permit the withdrawal of a number of 1.5 ml. samples was placed in a flask with a reflux condenser attached by means of a ground glass joint. The solution was refluxed and loss of water was thus kept to a minimum. The temperature of the boiling fluid in the flask could be assumed to be about 100° C. A considerable excess of solution was used so that its concentration would not be significantly affected by the slight amount of water that failed to run back down. Before withdrawing a sample, the neck of the flask and the lower part of the condenser were cooled with cold water from a wash bottle. In all of the experiments the pH of the fertilizin solutions was adjusted with the glass electrode just before heating was begun; a control sample was allowed to stand at room temperature throughout the total time of heating. As each sample was removed from the water bath or from the reflux flask,

TABLE V

Results of parallel titrations of heat-treated, purified fertilizin preparations of S. purpuratus

Fertilizin preparation	Initial pH	Sample	Temperature, degrees Cent.	Time in hours	Final pH	Titer		
						Sperm agglutination	Mucin clot	Inhibit
I*	7.1	a	96–98	0.5	—**	256	64	
		b	96–98	1.0	—	64	64	–
		Control	room	1.0	—	512	64	
II*	7.85	a	96–98	4.5	--	16	64	
		Control	room	4.5		256	64	
III*	7.4	a	96–98	3.5		128	256	0
		b	96–98	5.5	-	64	64	32
		Control	room	5.5		1024	256	
IV***	7.5	a	100	2.0	7.5	4096	512	
		b	100	3.0	7.2	4096	512	
		c	100	5.0	7.1	2048	256	
		d	100	6.0	7.1	2048	256	0
		e	100	7.5	7.1	1024–2048****	256	0
		Control	room	7.5	6.8	4096	512	-

* Individual 1.5 ml. samples heated in water bath.

** Dashes indicate not tested. Zero inhibition titer indicates tested but inhibition not detected.

*** Samples (1.5 ml.) withdrawn from refluxed solution.

**** A trace reaction probably present in the higher dilution.

it was placed immediately in the freezer. In the later experiments the pH of each heated sample was recorded before it was frozen. The titrations were performed as soon thereafter as possible. The results of the experiments are presented in Table V.

As may be seen from the table, reduction of sperm-agglutinating titer by heating is not necessarily accompanied by parallel reduction in mucin clot titer. Thus, for example, samples IIa and IIIa, heated for 4.5 and 3.5 hours respectively, showed no significant reduction in mucin clot titer although the sperm-agglutinating titer of the former was reduced to about 6 per cent and that of the latter to about 12 per cent of the original values. Sample IIIb was heated for 5.5 hours with a reduction of sperm-agglutinating titer to approximately 6 per cent of its original value. In this case the mucin clot titer was reduced to 25 per cent of the original value. Samples IVa–IVe show a more nearly parallel reduction of sperm-agglutinating and mucin clot titers than any of the others. Preparation IV was refluxed. After boiling for 7.5 hours the sperm-agglutinating titer was reduced to 25–50 per cent of the original value and the mucin clot titer was reduced to about the same per cent of the original value. Sample IIIb was the only one which gave an inhibition (univalence) titer. In the samples which were tested for inhibition but in which none was detected (IIIa, IVd, IVe), it is probable that insufficient univalent fertilizin was present in the high dilutions to permit detection. The inhibition test is unambiguous only in dilutions containing sufficient univalent fertilizin to react with most of the added sperm. In the high dilutions enough sperm remain uncombined to be agglutinated upon the addition of normal fertilizin and thus obscure the slight amount of inhibition that may be present. In the present experiments, sample IIIb was the only one in which sufficient univalent fertilizin was produced in the lower dilutions to give clear-cut evidence of inhibition. Since the inhibition titer of IIIb was 32, while the mucin clot titer was 64, it appears that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. Stronger evidence to support this view was afforded by experiments in which fertilizin was irradiated with ultra-violet light.

Parallel titrations with ultra-violet irradiated purified fertilizin preparations

Metz (1942) showed that univalent fertilizin is produced by irradiation of normal (multivalent) fertilizin by ultra-violet rays. In the present experiments ultra-violet irradiation was carried out in an apparatus consisting of glass tubing, 150 × 35 mm., fitted on the mid-section of a 15 watt General Electric "Germicidal" lamp, the diameter of which is 25 mm. The major part of the output of this lamp is concentrated in the 2537 Å wave-length band. The space between the outer wall of the lamp and the inner wall of the tubing is the irradiation chamber. The chamber and lamp assembly is mounted on a motor-driven rocker. An opening in the top of the chamber, which can be closed with a rubber stopper, permits the introduction and withdrawal of fluid. The chamber is cooled by means of a small electric fan mounted on the rocker platform; when the fan is in operation the temperature of fluid inside the chamber does not rise above 35° C. during irradiation.

In the first experiment, a purified fertilizin preparation of *S. purpuratus,* the pH of which was first adjusted to 7, was irradiated for a total of 2.5 hours. It was found, as shown in Table VI, that the sperm-agglutinating titer was reduced to

TABLE VI

Results of ultra-violet irradiation of purified fertilizin

Fertilizin preparation	Sample no.	Initial pH	Time of irradiation in hours	Final pH	Sperm agglutination titer	Mucin clot titer	Inhibition titer
I	Control	7.0	—	—	256	64	
	1	7.0	$2\frac{1}{2}$	—	4	32	–[1]
II	Control	7.67	—	6.60	1024	1024	
	1	7.67	$4\frac{1}{3}$	5.52	0	1024	128
	2	7.67	6	5.49	0	1024	128
	3	7.67	$7\frac{3}{4}$	5.49	0	512	4
	4	7.67	9	5.49	0	256	4

[1] Univalence present in this sample by inhibition test but titer not obtained.

about 2 per cent of the original value and the mucin clot titer was decreased to 50 per cent of the original. Tested by the inhibition method, the irradiated preparation was found to contain univalent fertilizin. The inhibition titer of this sample was not obtained. In a second experiment, a quantity of the fertilizin preparation which was found to be very heat-stable with respect to its sperm-agglutinating activity (preparation IV of the preceding section) was irradiated. The pH of the solution was first adjusted to 7.7. Small portions (1.5 ml.) were withdrawn at intervals up to nine hours; the first sample was removed after 4.3 hours of irradiation. A control sample was allowed to stand in natural light (filtered through window glass) at room temperature throughout the entire period of irradiation. Immediately upon the removal of each sample from the irradiation chamber, its pH was measured with the glass electrode, and then it was placed in the freezer. All of the samples, including the control, were stored in the freezer until the titrations could be performed. As shown in Table VI, all of the irradiated samples showed complete loss of agglutinating activity. Tested by the inhibition method, all of them were found to contain univalent fertilizin. The inhibition titers showed a progressive decrease as time of irradiation was increased. The mucin clot titers also showed a progressive decrease with increased time of irradiation. After nine hours the mucin clot titer was reduced to 25 per cent of its original value, and the inhibition titer was reduced to about 6 per cent of the value found after 4.3 hours of irradiation.

The results of these experiments demonstrate conclusively that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. They also show that ultra-violet irradiation is a more effective agent than heat in converting multivalent, purified fertilizin to the univalent condition. The progressive decrease in inhibition titer found in the second experiment indicates that degradation of the fertilizin by ultra-violet light proceeds beyond the stage in which it exhibits univalence.

DISCUSSION

In general it may be said that the mucin clot titer of untreated fertilizin preparations parallels their sperm-agglutinating activity. Sperm agglutination is usually detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

Destruction of the sperm-agglutinating activity of fertilizin is not necessarily accompanied by a reduction of mucin clot titer. Conversely, however, it is clear that fertilizin which has been subjected to treatment that causes a reduction in mucin clot titer, for example heating or irradiation by ultra-violet light for extended periods, will invariably show at least a parallel decrease in sperm-agglutinating activity. It has been shown in the experiments with ultra-violet irradiation that the capacity of the fertilizin to agglutinate sperm may be completely destroyed with but little, if any, loss of its ability to give the mucin clot reaction. The evidence shows that when the agglutinating (multivalent) form is degraded to the non-agglutinating (univalent) form, the latter continues to co-precipitate with protein in the mucin clot reaction. If a preparation in which all of the fertilizin has been made univalent is subjected to continued irradiation by ultra-violet light, a progressive decrease in both mucin clot and inhibition titers occurs.

The phenomenon of sperm agglutination by fertlizin has been interpreted by Tyler (1941, 1942, 1947, 1948) as an antigen-antibody type of reaction in which complementary combining groups of a substance (antifertilizin) on the surface of the sperm cells unite in "lock and key" fashion with the combining groups of fertilizin. Where a number of such combining groups are available on the surface of the fertilizin molecule, agglutination occurs as the result of the building up of a lattice, as postulated for analogous immunological reactions by Heidelberger (1938) and Marrack (1938). The formation of univalent fertilizin is brought about by various agents—e.g. heat, ultra-violet light, x-rays—which, according to Tyler (1941), split the molecule into fragments, each of which contains a single combining group. These fragments are still of large size, since they are non-dialyzable (Tyler, 1941). They are also capable of co-precipitating with protein in the presence of acid, giving the mucin clot reaction.

The ability to give the mucin clot reaction is, at least in the case of hyaluronic acid, presumably a function of the degree of polymerization of the molecule (Meyer, 1947). Depolymerized molecules are incapable of giving the reaction. Although fertilizin has been shown to be very different from hyaluronic acid in its chemical composition, the fact that it co-precipitates with protein in acid solution in an analogous manner suggests that it may be similar in its physical structure. Thus, fertilizin may normally exist in a polymerized condition. Sperm-agglutinating activity may, then, accompany a range of polymer size, and the univalent condition may represent a state of polymerization with which but a single combining element is associated. Degradation of multivalent fertilizin to the univalent form would then entail a progressive splitting off of relatively stable univalent units. The evidence from the experiments with ultra-violet irradiation indicates that the univalent form is in fact the more stable, since complete conversion to univalence was observed after 4.3 hours of irradiation, whereas even after nine hours both the inhibition and mucin clot titers retained significant values.

Summary

1. Preparations of fertilizin of three species of sea-urchin have been found to give a mucin clot reaction similar to that given by hyaluronic acid. Upon the addition of bovine serum albumin to an acidified solution of fertilizin, a precipitate forms which dissolves at a pH of 5.6 or higher. All of the sperm-agglutinating activity accom-

panies the precipitate and it is recovered quantitatively when the precipitate is dissolved.

2. A method for the determination of mucin clot titer of fertilizin is described.

3. At temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes by dialysis. Macroscopic aggregates appear, accompanied by a parallel decrease in mucin clot and sperm-agglutinating titers. Disappearance of the aggregates is accompanied by an increase in both titers.

4. Chemical analysis of fertilizin shows that it contains no glucuronic acid, about 2 per cent hexosamine and amino acids. Fertilizin, therefore, differs greatly from hyaluronic acid, but its ability to give the mucin clot reaction suggests an affinity with the class of mucopolysaccharides.

5. In general, mucin clot titer parallels sperm-agglutinating titer of the same untreated fertilizin preparation, although sperm agglutination is detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

6. Parallel mucin clot and sperm-agglutinating titrations were made with fertilizin preparations in untreated condition and after exposure to heat and to ultra-violet irradiation. The purified preparations used in these experiments proved to be exceptionally heat-stable; irradiation by ultra-violet light was found to be a more effective treatment in degrading the material.

7. Destruction of the sperm-agglutinating activity of fertilizin by heat and by ultra-violet irradiation does not necessarily cause a parallel decrease in mucin clot titer. The mucin clot reaction continues to be given by preparations in which all of the fertilizin has been converted from the normal, agglutinating condition to the non-agglutinating, "univalent" form. Continued irradiation of the univalent fertilizin is accompanied by a progressive decrease in both inhibition and mucin clot titer.

8. It is suggested that fertilizin may normally exist in a polymerized condition and that the non-agglutinating, "univalent" condition may represent a relatively more stable lower polymer of the native, agglutinating form.

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THE ANAPHASE MOVEMENT OF CHROMOSOMES IN THE SPERMATOCYTES OF THE GRASSHOPPER

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Among the many complex processes involved in the division of cells, the movement of the chromosomes at anaphase is most accessible to a causal analysis. The beautiful preciseness of the processes involved in the orderly separation of chromosomes has for a long time enticed biologists to search for their physico-chemical basis. However, before an analysis on this level is possible, it is necessary, first, to know what structural differentiations of the cell are involved, and secondly, to have detailed quantitative descriptions of the processes based on a study of living cells.

A previous analysis of chromosome movement in certain insects (Homoptera and Hemiptera) has shown that the structures involved in anaphase movement are the kinetochores on the chromosomes, the chromosomal fibers, which connect the kinetochores to the spindle, and the spindle body (Ris, 1943). The kinetochore determines the nature of the chromosomal fibers, which in the case of these insects are broad and sheet-like and attached to the entire length of the chromosome (cf. Hughes-Schrader and Ris, 1941). The movement of the chromosomes consists of two separate processes: first, the shortening of the chromosomal fibers, which moves the chromosomes to the poles of the spindle; and secondly, the elongation of the spindle body, which further separates the chromosomes. In the Homoptera and Hemiptera these two components of anaphase movement are separated in time, so that first the chromosomes move to the poles of the spindle and then, after a pause of a few minutes, the spindle body stretches and carries the chromosomes further apart.

In most animals and plants the chromosomal fibers are narrow bundles attached to a definite, restricted region of the chromosome. In this paper the spermatocyte divisions of the grasshopper were chosen in order to analyze chromosome movement in an organism with localized kinetochore.

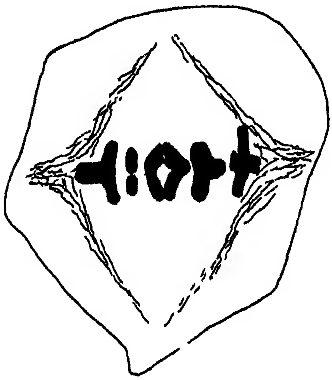
MATERIAL AND METHODS

The measurements recorded here were made on spermatocytes of *Chorthophaga viridifasciata*. A few measurements on *Dissosteira carolina, Melanoplus femur-rubrum, Arphia xanthoptera* and *Hippiscus spec.* gave similar results.

The spermatocytes of the grasshopper are classical material in the study of living cells in division (Chambers, 1914, 1924; Lewis and Robertson, 1916; Belar, 1929; Baumgartner and Payne, 1931). The usual technique consisted in breaking the testis follicles and spreading the cells on a coverglass in Locke's or Ringer's solution. Baumgartner and Payne (1931) showed that the follicles can be left intact and the cells studied with high powers. They pulled the testis through an opening

[1] Part of the work for this paper was done in the Department of Biology, Johns Hopkins University.

of the body wall into a little pool of salt solution, but left it attached to the vasa efferentia. Since they had to remove the follicular membrane which contains the trachae, the testes may as well be completely removed from the animal. In the present work the testes were dissected out, the follicular membrane removed, and the follicles spread intact on a coverglass into a drop of Belar's solution (Belar, 1929). The coverslip was inverted over a depression slide and sealed with paraffin. Aseptic technique was not attempted since only preparations made on the same day were used for measurements. The temperature was kept constant at 30° C with an electric stage warmer. A liquid filter of ferrous ammonium sulphate prevented heat from the lamp from reaching the object. The cells, thus, were disturbed as little as possible.



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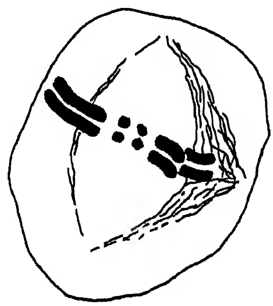

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FIGURES 1 and 2. Camera lucida drawings of living spermatocytes of Chorthophaga Side view of primary and secondary spermatocyte metaphases.

To measure the movement of chromosomes a metaphase in side view was selected, and as soon as the chromosomes began to separate, the distance between kinetochores was recorded at regular intervals with a camera lucida. In primary spermatocytes a bivalent with terminalized chiasmata near the spindle axis was chosen. In the secondary spermatocytes a chromosome in a median optical section of the spindle was selected. Though the spindle itself is hardly visible, it is clearly outlined by the chondriosomes (Figs. 1, 2). This makes it possible to measure the length and the equatorial diameter of the spindle during the entire anaphase. The various distances were then plotted against time, yielding a curve which describes the movement of the chromosomes and the changes in spindle length and diameter. All measurements were made with a 4 mm. Zeiss apochromat and 15 × ocular.

Observations

Anaphase movement in the first spermatocyte division

In Figure 3, three out of thirteen measured cells are presented.[2] The distance between separating kinetochores is plotted against time. The resulting curve con-

[2] The curves from different cells, even coming from different individuals, agree remarkably well, especially in the beginning of chromosome movement before spindle stretching sets in. The three cells shown indicate the degree of variation.

sists of an initial slow movement, then a straight portion of maximum velocity and a less regular part of gradually decreasing movement. Finally, before the cleavage furrow appears, the chromosomes move together again for a short distance, apparently due to the shrinkage of the spindle.

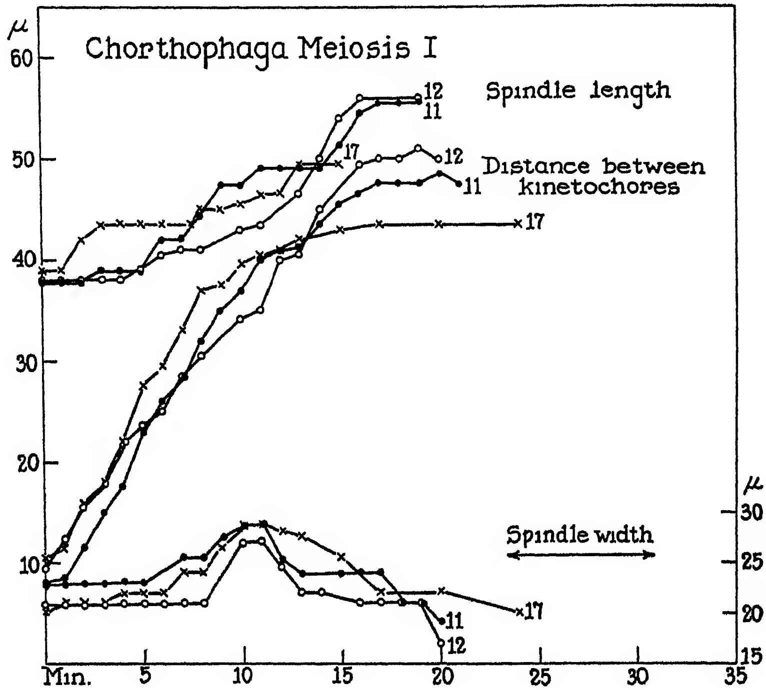


FIGURE 3. Chromosome movement and spindle behavior in the first meiotic division of *Chorthophaga viridifasciata*. See text.

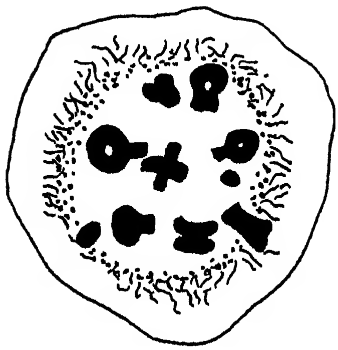
The spindle becomes visible in prometaphase through the alignment of the filamentous chondriosomes on its surface. In polar view their optical cross sections outline the spindle around its circumference (Fig. 4). In side view they appear lined up from the poles to the equator where they flow out into the equatorial plane (Fig. 1). Later, when the spindle elongates, the chondriosomes are stretched tightly on the spindle surface.

In Figure 3 the length of the spindle and its equatorial diameter are plotted against time during anaphase. We see how the spindle begins to elongate a few minutes after the onset of chromosome movement, contributing to their separation. There are thus two simultaneous processes involved in the later part of anaphase, namely, (1) the movement of the chromosomes to the poles due to the shortening of the chromosomal fibers, and (2) the elongation of the spindle. The diameter of the spindle increases in the later part of the chromosome curve, when the movement becomes irregular and slows down. Then it progressively decreases until the cleavage furrow cuts the spindle body in half. The spindle, therefore, increases

appreciably in volume during mid-anaphase. A comparison of the curves in Figure 3 shows that the spindle elongation varies more from cell to cell than the shortening of the chromosomal fibers. This indicates a greater sensitivity of that process to external conditions.

Anaphase movement in the second spermatocyte division

In the first division the chromosomes are distributed through the spindle body (Fig. 4). In the second division, however, they are oriented with their kinetochores at the periphery of a "hollow" spindle, the long arms pointing outwards (Fig. 5). Figure 6 gives the curves of three out of fifteen measured cells.[2] The



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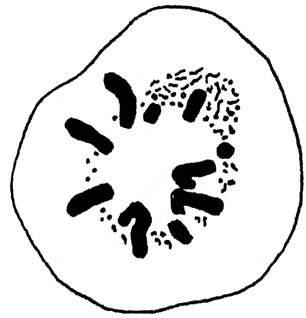


FIGURES 4 and 5. Camera lucida drawings of living spermatocytes of Chorthophaga. Polar view of primary and secondary metaphases.

movements of the chromosomes and the behavior of the spindle are much like those described for the first division. The chromosome curve is distinctly S-shaped. The spindle elongates a few minutes after the chromosomes have separated and increases in diameter in the later part of anaphase. Again there is thus a great increase in volume of the spindle. The rate of chromosome movement and spindle stretching is appreciably greater than in the first division.

In both divisions then we find the same type of anaphase movement. It begins with a shortening of the chromosomal fibers moving the chromosomes towards the poles. While this is continuing, the spindle begins to stretch, adding to the chromosome movement. Towards the end of anaphase the spindle begins to increase in width and then gradually shrinks until the cleavage furrow cuts it in half.

Experimental separation of the factors of anaphase movement

In the Hemiptera and Homoptera the action of chromosomal fibers and spindle elongation represent two distinct processes separated in time (Ris, 1943). In the grasshopper there is no such independence; the two processes are simultaneous and so neatly interwoven that a smooth movement of the chromosomes ensues. Is it possible to separate them experimentally? Methods have long been known which inhibit or destroy the spindle, such as ether, chloralhydrate, colchicine, etc. Is

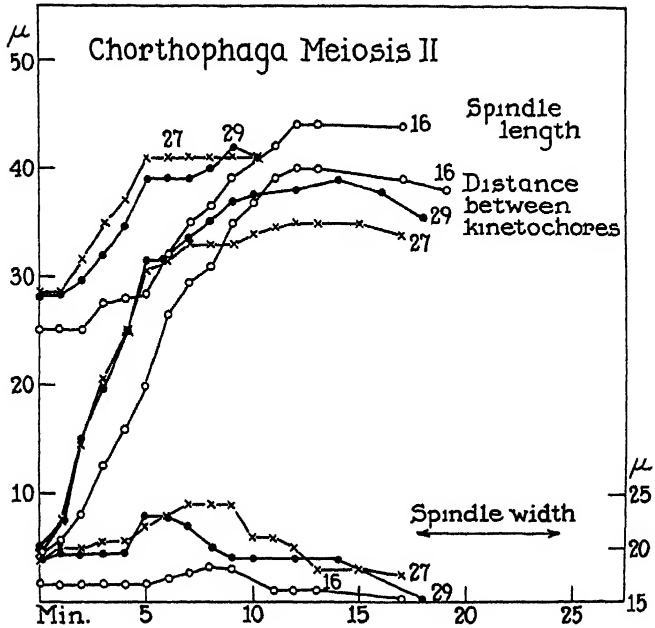


FIGURE 6. Chromosome movement and spindle behavior in the second meiotic division of *Chorthophaga viridifasciata*. See text.

there an agent which would inhibit one of the two processes without affecting the other? Colchicine, if added to the medium, either destroyed the spindle completely, or in lower concentrations had no effect on anaphase movement. Chloralhydrate, on the other hand, proved more useful. In concentrations higher than 0.1 per cent the spindle became shorter and narrower, and finally disappeared. The chromosomes were scattered irregularly through the center of the cell. The chondriosomes lost their regular orientation and began to penetrate between the chromosomes. At a concentration of 0.08 per cent cells were found in which the chromosomes moved to the poles, but where spindle elongation was inhibited. This seems to happen only within narrow limits of concentration of chloralhydrate inside the cell. If there is too much, the spindle will break down; if there is too little, it will elongate normally. This critical concentration is usually obtained only in a few cells of one cyst. The distance between chromosomes, and the length of the spindle, were then recorded during anaphase in primary spermatocytes exposed to chloralhydrate. In Figure 7 two such curves are shown (36 and 37). The spindle remained the same length all through anaphase in cell 37 and became only slightly longer in cell 36. The chromosomal fibers, on the other hand, must have remained active since the chromosomes had moved to the poles in a regular fashion. It can be shown that this action of the chromosomal fibers is normal. If we subtract the spindle elongation from the chromosome curve of an untreated primary spermatocyte, we obtain a curve which represents the movement of the chromosomes due to the chromosomal fibers alone. Two such curves are plotted in Figure 7 (13a and 17a). Since they agree well with the experimental curves (36 and 37), we must conclude that the

action of the chromosomal fibers was not affected by the chloralhydrate even though the spindle was prevented from elongating.

In the Hemiptera and Homoptera the two factors of anaphase movement, contraction of the chromosomal fibers and elongation of the spindle body, are separated in time. In the grasshopper they overlap in time, but their differing sensitivity to chloralhydrate has made it possible to separate them experimentally, inhibiting

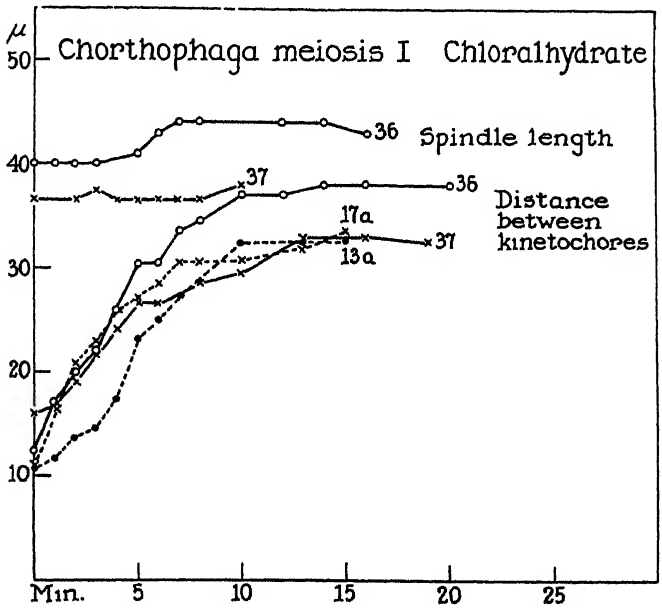


FIGURE 7. Chromosome movement and spindle elongation in Belar's solution with 0.08 per cent chloralhydrate (curves 36 and 37). Curves 13a and 17a represent the normal chromosome movement after the spindle elongation has been subtracted. The four curves are similar, showing that the movement to the poles in the absence of spindle stretching is normal.

spindle elongation without affecting the contraction of the chromosomal fibers. The difference between the grasshopper and the Hemiptera and Homoptera lies mainly in the relative timing of the component processes. In the grasshopper, spindle elongation sets in before the chromosomes have reached the poles. In the Hemiptera and Homoptera the spindle does not stretch until a few minutes after the poleward movement of the chromosomes has been completed.

Recently Callan (1941) described a case in which a separation of the components of anaphase movement occurs under natural conditions. In a trisomic grasshopper (Mecostethus) the unpaired extra chromosome sometimes moves into the equatorial plane during the first meiotic anaphase. In these cases the spindle does not elongate. The poleward movement of the chromosomes, however, does not seem to be disturbed. For reasons unknown, spindle elongation is inhibited under these conditions while the chromosomal fibers do not seem to be affected. Of course, we do not know here whether the rate of movement is normal as was shown in the chloralhydrate experiments.

The effect of temperature on the anaphase movement of chromosomes

The effect of temperature on mitosis has been repeatedly investigated, in most cases, however, on over-all processes such as the length of the mitotic phases, the rate of cleavage, etc. (see Belehradek, 1935). Only little can be concluded from such studies unless the processes are broken down into their components and the effect of temperature on these components analyzed. The effect of temperature on chromosome movement in living cells was studied by Bucciante (1927) in chick fibroblasts and by Barber (1939) in Tradescantia stamen hair cells. They found an increase in the rate of chromosome movement with rising temperature. This increase was large at lower temperatures and small at higher temperatures. In chick fibroblasts there is a maximum rate at 40° C. Fauré-Fremiet (1925) had earlier reported an optimum temperature (37° C.) for cell division in Ascaris. Measurements of chromosome movement in the secondary spermatocyte of the grasshopper at 17°, 23° and 30° C. agree with these findings (Fig. 8 and Table 1). The data for Tradescantia (Barber, 1939) and grasshopper also suggest an optimum temperature, though the range of temperature was not wide enough to show the decrease at higher temperatures. In the grasshopper a temperature above 32° C. destroys the spindle and thus inhibits chromosome movement

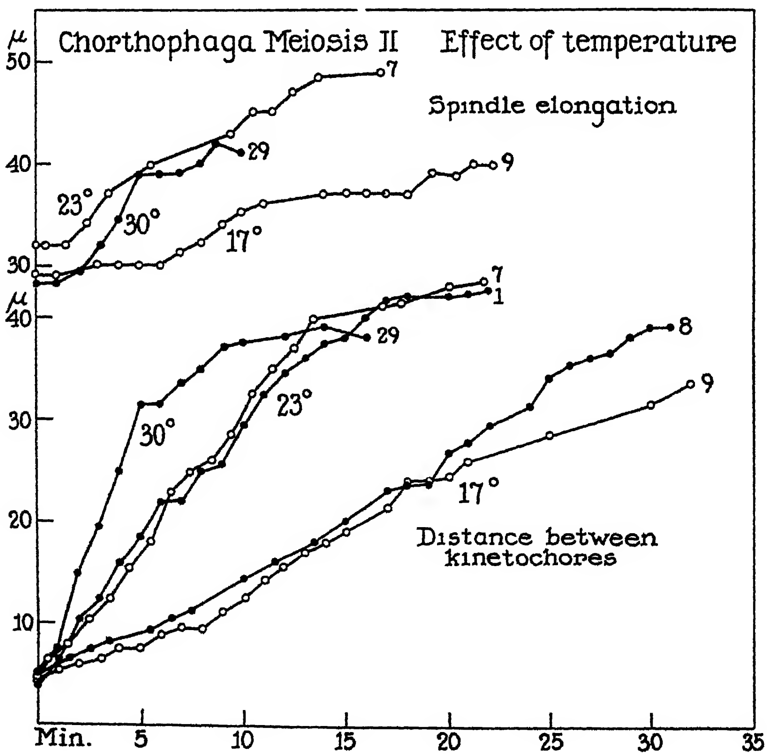


FIGURE 8. Chromosome movement and spindle elongation at 17°, 23°, and 30° C.

TABLE I

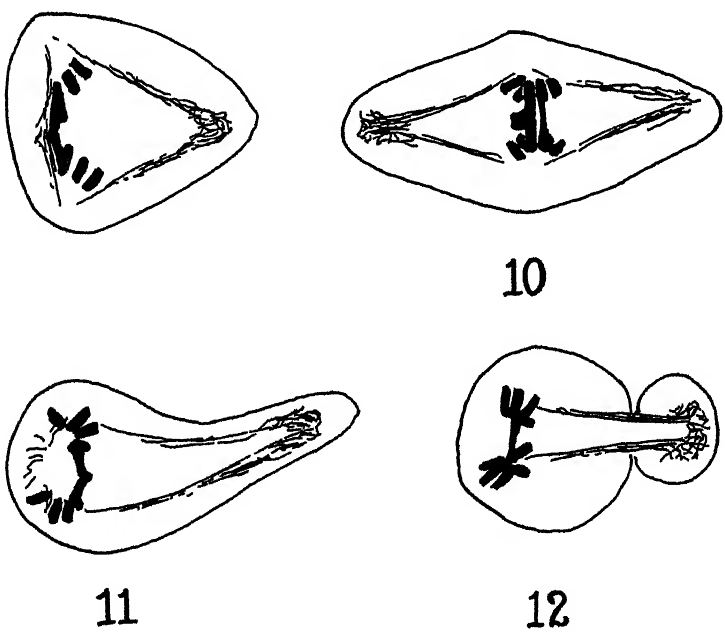
Effect of temperature on the rate of chromosome movement (chromosomal fibers only), and on the rate of spindle elongation. Micra/minute

Temperature	Maximum velocity chromosomes	Maximum rate spindle elongation
17° C.	0.4	1.4
23°	1.2	2.4
30°	2.5	3.6

Exposure to low temperature (1°–5° C.) destroys the mitotic spindle, as has been known since the experiments of O. Hertwig (1890) on sea urchins. If grasshopper spermatocytes at metaphase are exposed to 1° C., the spindle disappears, the chondriosomes become arranged at random, and the chromosomes are dispersed through the former spindle area. The cells can remain in this state for hours. If they are again exposed to a higher temperature (30° C.) the spindle forms anew, the chondriosomes are lined up on its surface and the chromosomes arranged in the metaphase plate. This process can be repeated several times on the same cells.

Abnormal spindle elongation

The increase in volume during anaphase is a characteristic property of the spindle in most animal cells. This swelling manifests itself especially in a pronounced elongation which contributes to the anaphase separation of the chromosomes that are attached to it by means of chromosomal fibers. As was shown above,



FIGURES 9–12. Diagrams demonstrating the abnormal lateral stretching of the spindle in primary spermatocytes after X-ray-induced sticking of the chromosomes.

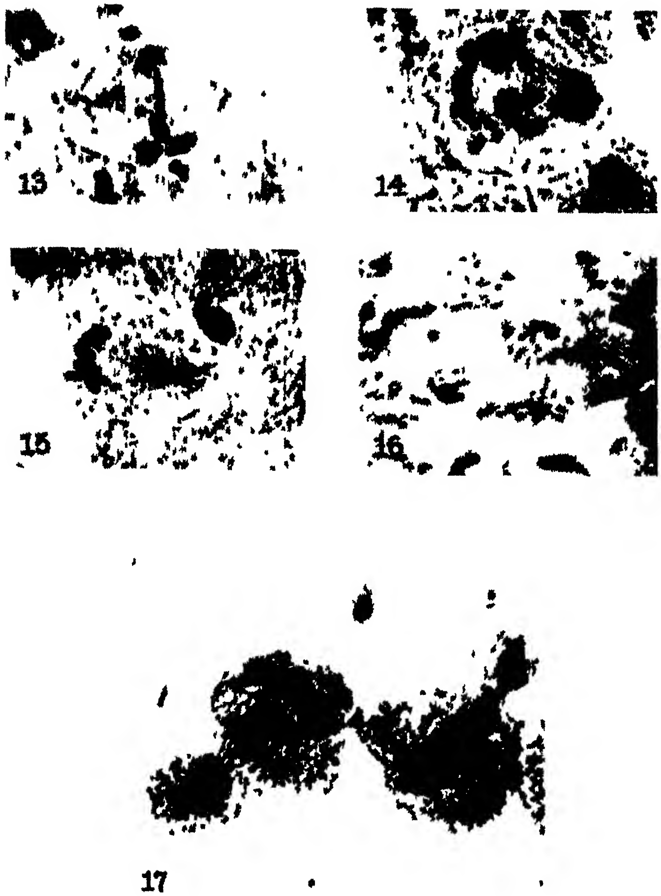
the spindle also increases in width in mid-anaphase and then gradually shrinks until it gets pinched through by the cleavage furrow. This stretching ability of the spindle is especially impressive under certain abnormal conditions. If the first meiotic division is observed in living cells after X-raying, in hypertonic medium or at temperatures around 32° C., one finds that chiasmata have a tendency to stick so that bivalents can not separate at anaphase. When the spindle begins to stretch, its normal elongation in the polar axis is inhibited by the combination of chromosomal fibers and sticking chromosomes. The spindle then begins to bulge in the equator, opposite the sticking chromosomes (Figs. 9, 14). If several bivalents fail to separate, the spindle may bend outward in several places (Figs. 10, 16). With the further elongation of the spindle, these lateral bulges become long and narrow projections which begin to push out the cell membrane. The poles of the spindle approach each other during this process, probably because of the action of the chromosomal fibers, which, instead of pulling the chromosomes to the poles, now draw the poles closer together (Figs. 11, 15). In fixed and stained preparations, the course of the continuous fibers shows clearly that the lateral projections are parts of the spindle bending outwards (Figs. 13–15). If chromosomes stick only on one side of the spindle, a very characteristic bent spindle results, looking like a spindle folded in the middle. Actually the origin is quite different, as described above. Sometimes the sticking chromosomes separate in mid-anaphase. The spindle then is able to assume its normal shape. It elongates in the polar axis and the lateral bulges disappear.

Spindle elongation and cleavage furrow

The relation of spindle elongation to cytoplasmic division, as demonstrated by these abnormal anaphases, is of special interest. When the spindle does not elongate normally, the cleavage furrow is always delayed or does not appear at all. More striking are the cells in which the spindle has been forced to elongate laterally in the equatorial plane. The lateral bulges of the spindle begin to push the cell out into long narrow processes (Figs. 11, 14). At the time when normally the cleavage furrow is formed, constrictions become visible around these cell projections. Often these constrictions develop into regular cleavage furrows and pinch off one or more small anuclear buds (Figs. 12, 17). In the cysts with secondary spermatocytes, one finds then cells with the diploid number of chromosomes which undergo the second division, and anuclear buds which do not divide any more. In grasshopper spermatocytes the cleavage furrow is therefore dependent on cell elongation caused by the stretching of the spindle. The location of the furrow is not predetermined, but can occur wherever the cell is pushed out.

Time relations

The main difficulty in the timing of the phases of mitosis, particularly in living cells, is the separation of the process into clearly delimited sections. The usual separation into prophase, metaphase, anaphase and telophase is not well suited for this purpose since the beginning or end of these phases is usually without sharp boundary. The duration of the following well-marked phases was measured in the spermatocytes of the grasshopper at 30° C.: First division: (1) Metakinesis, from



FIGURES 13–15. Anaphase in primary spermatocytes of Chorthophaga after irradiation with X-rays (100 r). Note the sticking of chromosomes and the lateral expansion of the spindle. Fixation: Sanfelice; stain: Iron-hematoxylin. 4 mm. Zeiss Apochromat, 15 × ocular. Compare with Figures 9–12.

the disappearance of the nuclear membrane to the formation of the metaphase plate. (2) Metaphase, from the establishment of the metaphase plate to the beginning of anaphase separation. (3) Ana-telophase, from the beginning of chromosome (kinetochore) movement to the appearance of the nuclear membrane. Interphase: from the formation of the nuclear membrane to its breakdown in the secondary spermatocyte. Second division: same phases as in the first division. The processes which mark these stages are clearly visible in the living cell. In the first division the asters are visible mainly due to the radial arrangement of the chondriosomes, but sometimes astral rays can be seen. The nuclear membrane, which was sharply outlined in prophase, becomes irregular and wrinkled, then disappears first near the asters. Wrinkled remnants can be seen for a few minutes before they vanish.

The metaphase spindle then slowly takes shape after the orientation of the asters. Its outline is marked by the chondriosomes. The spindle is at first rather narrow and short (cf. Belar, 1929, p 433). The chromosomes are thus crowded into the middle of the cell. Then, while the chromosomes become arranged into the metaphase plate, the spindle increases in width and in length. During metaphase the spindle remains constant in size and varies little from cell to cell. In telophase the nuclear membrane appears around a light area containing the chromosomes. The nucleus then enlarges until the regular interphase size is reached. In the second division the same processes are repeated, except that no asters can be seen in living cells. Table II gives the duration of these phases. Cells 1 and 2 were followed through the two divisions, cells 3 and 4 only through part of meiosis. Cells 3 and 4 are from a different individual than cells 1 and 2. At constant temperature the length of each phase varies only slightly from one individual to another.

TABLE II

Time relations in the meiotic divisions of the grasshopper Chorthophaga (30° C.) (hours and minutes)

	Cell 1	Cell 2	Cell 3	Cell 4
I. Metakinesis	50	—	43	-
I. Metaphase	2/30	2/45	2/40	
I. Anaphase Telophase	1/25	1/40	1/25	-
Interphase	2/15	2/40	—	—
II. Metakinesis	25	—	—	25
II. Metaphase	1/25	1/25	—	1/35
II. Anaphase Telophase	1/45	1/47	—	1/45

DISCUSSION

The causal analysis of mitosis strives to dissect the complex process of cell division into its component factors and to elucidate their composition and their mode of action. Since Belar's classical study, the structures involved in the mitotic movements have again received deserved attention. In the grasshopper we can distinguish the following mitotic organelles: center, kinetochores, chromosomal fibers, spindle body.

The center

Like most animal cells the grasshopper spermatocytes contain a pair of centrioles which move to opposite sides of the nucleus in prometaphase and form the poles of the developing spindle. The asters are rather inconspicuous as in other cells with relatively little cytoplasm. In living cells they can be seen in prophase and occasionally in metaphase, especially in a hypertonic medium (cf. Belar, 1929). In secondary spermatocytes asters are even less distinct. Little is known about the function of the centers, except that they are probably involved in the organization of the spindle and in the cytoplasmic streaming which goes on during metaphase and anaphase.

Kinetochores

This specialized region of the chromosome is essential for the regular movements within the spindle. Fragments which are devoid of it lag behind and do not show any regular orientation, but may be moved passively by the stretching spindle or cytoplasmic currents on the surface of the spindle (White, 1935, 1937; Carlson, 1938). The main function of the kinetochores, perhaps in cooperation with the spindle or centriole, appears to be the formation of chromosomal fibers Without kinetochores no chromosomal fibers can be formed.

Chromosomal fibers

Soon after the nuclear membrane has disappeared and the spindle begins to take shape, we can find in fixed and stained cells a distinct fibrous connection between the kinetochores and the spindle poles. These are the chromosomal fibers. They are usually not visible in living cells and some investigators therefore deny their existence.[3] Yet there is enough circumstantial evidence to show that they exist as differentiated structures within the spindle, and that they are the major factor in the anaphase movement of chromosomes (cf. Cornman, 1944; Schrader, 1944).

Belar (1929) emphasized the role of these "traction fibers." He assumes that they originate as a fluid secretion by which the kinetochore attaches itself to a fiber of the spindle body ("Leitfaser") and which allows the chromosome to glide along this "Leitfaser" in anaphase. Schrader (1944) accepts this view of Belar and bases on it his classification of spindles. Yet, the present writer could find no evidence for this indirect formation of the chromosomal fibers. They appear in prometaphase even before the spindle is fully formed as direct connections to the centers. They anchor the chromosomes to the poles of the spindle. So, when the spindle elongates, the chromosomes are carried along, the pull being transmitted through the chromosomal fibers to the kinetochores. If a chromosome sticks at anaphase, it will prevent the spindle from elongating on that side. The combination chromosome-chromosomal fibers is thus stronger than the spindle, while the cell membrane, for instance, yields to its pushing force. The chromosomal fibers, when they contract at anaphase, can even pull the spindle poles together and force the spindle out to one side of the cell (Figs. 9–12). The chromosomal fibers must thus be of greater consistency than the spindle body.

The spindle body

In grasshopper spermatocytes the spindle body develops from nuclear material between the two centers. The area around the chromosomes remains distinct even after the nuclear membrane has disappeared, and stays free of cytoplasmic inclusions like chondriosomes. In metaphase the spindle is a viscous body which can be moved about and dissected out by microneedles (Chambers, 1924). It appears homogenous in the living cell and fibrous after fixation. The spindle is essential for the orientation of chromosomes and for the action of chromosomal fibers since they are anchored at its poles. The spindle can be destroyed by a number of agents: colchi-

[3] Chromosomal fibers are sometimes visible in forms with diffuse kinetochore, if the chromosomes are viewed on end and the light therefore has to pass the entire length of the sheet-like chromosomal fibers (Hughes-Schrader and Ris, 1941; Ris, 1942).

cine, chloralhydrate, cold, heat, hypertonic medium, etc. At the same time the regular arrangement of the chromosomes disappears and all chromosome movements are stopped.

The most striking action of the spindle is the elongation during anaphase. Belar (1929) found that in hypertonic media this elongation appears to be greatly exaggerated, and this led him to a very detailed study of spindle stretching in hypertonic solutions. His conclusions are briefly: (1) The spindle has a tendency to stretch; this tendency is exaggerated in hypertonic media. (2) The spindle, by origin, is differentiated into two half spindles, the "Stemmkorper" (pushing body) developing at anaphase between the daughter plates. (3) At anaphase, it is the "Stemmkorper" in particular which elongates.

As was shown above, abnormal spindle stretching occurs not only in dehydrated cells, but always when daughter chromosomes are made to stick together. A hypertonic medium is just one way of causing chromosomes to stick at anaphase. This effect of hypertonic solutions on chromosomes was described by Konopacki (1911) in cleavage divisions of echinoderm eggs, by Kostanecki (1898) in Myzostoma, and by Moellendorff (1938) in tissue cultures. Similar accidents are found at high temperatures (over 30° C.) and after exposure to X-rays. It is therefore not the hypertonic medium which induces the abnormal spindle stretching, but the resistance to elongation in the main axis, brought about by the sticking of chromosomes. From Belar's figures, it is obvious that in primary spermatocytes all the abnormal spindles are correlated with sticking chromosomes. The bent spindles in secondary spermatocytes are of a different and less extreme kind (Fig. 45, Belar, 1929). Here it seems to be the cell membrane which offers resistance to the elongating spindle and causes it to bend. X-ray-induced bridges cause the same kind of abnormal spindles in secondary spermatocytes as Belar described in the first division. Lateral expansion of the spindle after X-ray-induced chromosome sticking was also figured by White (1937, Figs. 12, 13).

But Belar figures some cells which show exaggerated elongation of the spindle without sticking of chromosomes. These cells had been treated in anaphase. During anaphase the spindle increases not only in length, but also in volume. Belar believed that the volume remained constant, though he did not commit himself definitely. His beautiful drawings, however, indicate quite clearly the swelling of the spindle which measurements have now substantiated. In hypertonic solutions the cell shrinks greatly, and as Belar pointed out, the cytoplasm more so than the spindle. The swelling of the spindle, then, encounters resistance, and it is probably this factor which causes the spindle to be longer, but narrower than normally. Even so, these spindles are only found in free floating cells. In this writer's preparations where the cells remained in the follicles, they did not occur. Another case of spindle stretching without chromosome sticking is found if prometaphases are treated with hypertonic solutions (Belar, 1929, Fig. 55; Ris, 1942, in spermatocytes of the bearberry aphid). Here again the spindle increases in width during its formation. In dehydrated cells this lateral growth is interfered with, and the spindle becomes long and narrow. In this connection it is important to note that the volume of the abnormally stretched spindles appears to be not larger than in normal spindles. There is, therefore, only a distortion in shape, not an actual increase in the spindle material.

Two factors then cause abnormal spindles: interference with the increase in width in prometaphase and mid-anaphase, and interference with normal stretching during anaphase through the sticking of chromosomes

From his studies of these abnormal spindles, Belar came to the conclusion that the part of the spindle between the separating chromosomes was mainly responsible for the stretching. He called it the "Stemmkorper" (pushing body) and distinguished it from the two half spindles between the chromosomes and poles. This subdivision of the spindle is, however, artificial and unjustified. Belar himself points out the uniformity in the aspect of the entire spindle. Fibers and clefts are continuous. The only difference in anaphase is the presence of chromosomal fibers in the cone-shaped region between the chromosomes and the poles. This is responsible for the darker appearance after staining. In Belar's Figure 41 the chromosomal fibers are especially clear. The "Stemmkörper" concept originated in the observation that the region between the daughter plates elongates more rapidly than the entire spindle. This appears so, not because this region is a special part of the spindle, but because the chromsomal fibers actually shorten during spindle elongation, pulling the chromosomes to the poles. In this way the impression of a special stem body between the daughter plates is produced.

Furthermore, Belar thought that the initial separation of the chromosomes through action of the traction fibers releases the tension in the spindle and originates the action of the "Stemmkorper." But actual timing has now shown that the chromosomes travel a good distance to the poles before the spindle elongates. Besides, spindle stretching can occur without any action of the chromosomal fibers as is shown in the first spermatocyte division of Tamalia. The chromosomal fibers act merely as passive anchors for the chromosomes (Ris, 1943). In the lepidopteran Orgyia the spindle elongates though the chromosomes have no chromosomal fibers at all (Cretschmar, 1928, Figs. 48–50). All the evidence then indicates that there is no differentiation into "half spindles" and "Stemmkörper." The only real differentiations are the chromosomal fibers and the spindle body. The chromosomal fibers pull the chromosomes to the poles. The stretching of the spindle has nothing to do with this phase. It can go on just as well without spindle elongation (chloralhydrate experiment). But spindle elongation has its important functions. It separates the daughter plates still further by pushing the poles apart and thus indirectly moves the chromosomes anchored to them.

The picture of anaphase movement in grasshopper spermatocytes presented here is essentially in agreement with Belar's view. There is a "pulling action" of chromosomal fibers and the stretching of the spindle. But there are some modifications. The subdivision into half spindles and "Stemmkörper" is found to be artificial. The spindle as a whole elongates, at the same time increasing in volume. Its action on the chromosomes is indirect, through the chromosomal fibers which connect them to the spindle poles.[4] The chromosomal fibers are thought to connect the kinetochores directly to the poles without the intervention of a "Leitfaser." They shorten during anaphase and are alone responsible for moving the chromosomes to the spindle poles.

[4] Just how the chromosomal fibers are attached to the spindle is a very puzzling problem and nothing definite can be said about it at present.

In addition to being a major factor in the movement of chromosomes, the spindle body also seems to play a role in the division of the cytoplasm. If the spindle does not elongate, as in the chloralhydrate experiments, no cleavage furrow is formed. When the spindle stretches laterally instead of in its long axis, a cleavage furrow does appear at a right angle to this elongation in a quite unorthodox position and produces an anuclear bud (Figs. 9–12). Bauer (1931) illustrates a similar situation in spermatocytes of Tipula with abnormal spindles. His Figure 23 h suggests that it originated in the same fashion. Many examples can be found in the literature which show how the failure of spindle stretching causes absence of the cleavage furrow (for instance Dobzhansky, 1934; Callan, 1941). In most plant cells there is little or no stretching of the spindle and the cytoplasm is divided by the formation of a cell plate. But in the pollen mother cells of some plants a cleavage furrow is formed, and it is then associated with elongation of the spindle (Guignard, 1897; Farr, 1918). It appears then that in dividing cells, elongation of the cell and cleavage furrow are associated with spindle elongation (in contradiction to the unwarrantable generalization of Buchsbaum and Williamson, 1943). Dan has recently (1943) assembled convincing evidence that spindle elongation is the active agent in cell elongation and the following formation of a cleavage furrow.[5] On the other hand, in certain abnormal cases the spindle elongates and yet no cleavage furrow appears. The formation of a cleavage furrow clearly depends on other factors in addition to spindle elongation.

In a recent paper, Hughes and Swann (1948) published chromosome separation and spindle elongation curves for chick embryo cells in tissue culture. Chick chromosomes possess a localized kinetochore and the achromatic apparatus is similar to that of the grasshopper spermatocytes. The anaphase movement as described by the curves of Hughes and Swann is essentially the same as we found in the grasshopper. Their curves show spindle elongation to start right from the beginning of anaphase, while in the grasshopper it does not begin until the chromosomes have moved a considerable distance. This is probably not a real difference but the result of the great difficulties involved in making measurements in early anaphase on the small chromosomes and spindles of the chick embryo cells.

We have set out to describe the movement of chromosomes during anaphase in terms of the mitotic organelles involved. The structures responsible for this chromosome movement were found to be the chromosomal fibers and the spindle body. The chromosomal fibers move the chromosomes to the spindle poles by decreasing in length. The spindle body swells and stretches and moves the daughter chromosomes further apart, since they are anchored to the spindle by the chromosomal fibers. If these are broad sheets attached to the entire length of the chromosome (diffuse kinetochore), the spindle does not elongate until the chromosomes have reached the spindle poles (hemipteran and homopteran insects). If the chromosomal fibers are narrow bundles attached to a very short region of the chromosome (localized kinetochore), the spindle begins to stretch shortly after the chromosomes have begun to move. The two processes then act simultaneously producing a smooth unbroken chromosome separation curve. Though we cannot see here directly how the two factors act on the chromosomes, we can separate them experi-

[5] I am indebted to Dr. D. Costello, University of North Carolina, for making this paper available to me.

mentally by inhibiting spindle elongation with chloralhydrate. It is then possible to study the action of the chromosomal fibers alone.

Very little is known about the nature and mode of action of these organelles, and this aspect will not be discussed here. Many more exact data on the structure, composition, and behavior of spindle and spindle components under various conditions are needed before a fruitful hypothesis on the physico-chemical level can be brought forward.

Summary

The movement of chromosomes and the changes in spindle size have been recorded in living spermatocytes of the grasshopper during the meiotic divisions. Anaphase movement consists of two separate processes which are related to the action of distinct cellular organelles: (1) The shortening of chromosomal fibers moves the chromosomes to the poles. (2) The elongation of the spindle further separates the daughter plates. The two processes act simultaneously in the grasshopper. With chloralhydrate, spindle elongation can be inhibited without affecting the action of the chromosomal fibers. This demonstrates the independence of these two factors.

The effect of temperature on chromosome movement is shown by measurements at 17°, 23° and 30° C. Between 17° and 23° there is a greater increase in velocity of chromosome movement than from 23°–30° C. Temperatures above 32° C. inhibit mitosis through the destruction of the spindle.

Abnormal spindle elongation is found whenever chromosomes stick at anaphase. The spindle, unable to elongate in its long axis, expands laterally into a disc-shaped body which later forms one or several finger-like processes, pushing out the cell membrane. These lateral elongations usually give rise to one or more cleavage furrows, pinching off one or, rarely, more anuclear buds. This demonstrates clearly the relationship between spindle elongation, cell elongation, and cleavage furrow.

The role of the mitotic organelles in the anaphase movement of chromosomes is discussed. Indispensable for a regular anaphase are the kinetochores on the chromosomes, the chromosomal fibers, and the spindle body. No evidence was found for a specialized region in the spindle acting as "Stemmkörper." The spindle is uniform in structure and elongates uniformly.

Distinct recognition of the structures involved in anaphase movement, and a quantitative description of their function, forms a basis for experimental analysis of their composition as well as their mode of action.

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TEMPERATURE COEFFICIENTS OF RESPIRATION IN PSAMMECHINUS EGGS

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INTRODUCTION

In *Arbacia punctulata* Rubenstein and Gerard (1934) using Warburg technique, found a Q_{10} of oxygen consumption for fertilized eggs of 1.8 between 13 and 30° C., whereas unfertilized eggs had a much higher value, viz., 4.1. These remarkable findings were principally confirmed by Korr (1937), who extended the experiments (Warburg technique) and discussed the results from biochemical and physiological points of view.

On the other hand Tyler and Humason (1937), working on *Strongylocentrotus purpuratus* (Warburg technique), found no significant difference in respiration Q_{10} between fertilized and unfertilized eggs in the temperature range 5–22° C. So, for example, the Q_{10} values for 10–20° C. were 2.3 and 2.6 respectively. Similar results were reported for Dendraster, Ciona and Urechis.

In earlier investigations (Warburg technique) by Ephrussi (1933) on *Paracentrotus lividus* the same Q_{10} of oxygen uptake was found between 14.9 and 22.4° for unfertilized eggs and gastrulae, viz., $\sim$ 2–2.5. At lower temperatures there was a tendency to slightly higher Q_{10} values in the eggs. Loeb and Wasteneys (1911) using Winkler technique reported very low values for unfertilized Arbacia eggs ($Q_{10} \sim$ 1.3 between 5 and 25° C.), but normal values for fertilized eggs ($Q_{10} \sim$ 2–2.5 for the range 3–25° C.).

In view of the probable significance of temperature coefficients of respiration for elucidating the different oxidative mechanisms of fertilized and of unfertilized sea-urchin eggs, it was thought of importance to investigate the matter in another species. Recently Borei (1948a) studied the respiration of eggs of *Psammechinus miliaris* before and after fertilization. Because of the facts already known about this species, it was chosen for the present investigation.

Experimental Data

The experiments have been performed with Cartesian diver micro-respiration technique, suitable for measurement of the oxygen consumption of ~ 100 eggs at a time. Concerning material and methods, corresponding chapters (2.1–2.3; 3.111; 3.114) in Borei (1948a) should be consulted. Diver charge type I (Borei, 1948b) was used throughout.

The temperature range was 10–21° C. The maximum temperature for normal larval development of *Psammechinus miliaris* has been studied by S. Runnström (1927), who found it to be 22° C.; the minimum temperature was found by this author to be 8° C. For measurements at lower temperatures, the cooling coil of the diver apparatus thermostat was fed with refrigerated salt-water of approximately + 5° C. The desired temperature was obtained by counteracting the cooling device by operating a thermostatically controlled electric heating bulb.

In order to obtain more comparable values, measurements were only performed on the flatter part of the declining respiration curve of the unfertilized egg (cf. Borei, 1948a, Chapter 3.112.1). Thus the time of actual measurement usually ran from three to six hours after removal from the ovary. This means that the constant part of the respiration is dominant during the measurements, whereas the "rapidly declining" part characterizes the preceding 2.5–3 hours, during which the eggs were kept at 16–18° C. The eggs were, on an average, placed in the diver 2.5 hours after removal from the ovary. The diver was then immediately placed in the thermostat at the experimental temperature and left there for a half hour for temperature equilibration before starting the measurements. Usually two diver thermostats were operated simultaneously, thus allowing measurements at two different temperatures. The time schedule of the experiments may be seen from Figure 1.

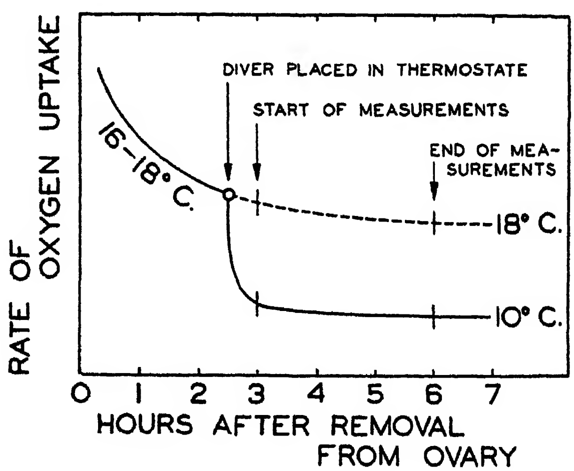

Figure 1. Average time schedule of experiments.
An actual experiment with measurements at 10° C. and a control experiment at 18° C. is assumed in the figure.

After completed diver measurements, the cells were washed out of the divers with sea-water, re-counted and then microscopically observed as to condition and fertilizability. Only those experiments were accepted in which the cells passed these post-diver measurement controls satisfactorily.

Previous authors have stated their results in rates of oxygen consumption per volume of cell matter. Great pains were taken by them to estimate the volume accurately. Probably the most correct way will be that adopted by Korr (1937): egg volume obtained by multiplying the number of eggs by the average egg volume. Technical difficulties were met, however, in estimating the exact number of eggs. In the present investigation neither the counting of the eggs nor the measurement of the average size of the eggs will give any difficulties. In view of these facts and of the great variability of the cell diameter, it has been thought more advisable, even in this investigation, to state the rate of oxygen uptake on a cell volume basis. This procedure also permits of direct comparison with the results of the previous authors.

In order to obtain a measure of the cell volume, the cell diameter was estimated of a number of eggs (> 20) from every female used, by means of a calibrated ocular micrometer (cf. Borei, 1948a, Chapter 3.114).

TABLE I

Oxygen consumption at different temperatures of unfertilized Psammechinus miliaris eggs

Volume of oxygen, measured at 0° C. and 760 mm. Hg, consumed per volume of cell matter and hour. All measurements are on egg material from S-form animals, except those marked *, which are on material of the Z-form.

Temperature (° C)	Oxygen consumption
10	0.024 0.053 0.069
11	0.062 0.066 0.069
12	0.067
13	0.056 0.076
14	0.078 0.083
15	0.060 0.113
16	0.033 0.046 0.099 0.112
17	0.061 0.076 0.088
18	0.063 0.065
18	0.080 0.082 0.084 0.099 0.107* 0.132 0.151* 0.160
19	0.086 0.089 0.140 0.145 0.173 0.188
20	0.065 0.111 0.116 0.126
21	0.090 0.122* 0.131 0.172*

The oxygen consumption figures obtained are referred to 0° C. and 760 mm. Hg, in order to render them intercomparable, irrespective of the actual temperature of measurement. Corrections are introduced according to Figure 2 of Borei (1948a) for deviations from the time schedule of the above Figure 1.

The results of the experiments are given in Table I.

The average cell volume, calculated from the separate figures used for the evaluations in Table I, is 5.89×10^{-4} µl. per egg (n = 44). (Corresponding value given by Borei, 1948a, = 5.84×10^{-4}.) The average oxygen consumption rate at 18° C. was found in the experiments (n = 10) to be 0.51×10^{-4} µl. per cell and hour. (Corresponding value given by Borei, 1948a, = 0.53×10^{-4}.)

Discussion

Rubenstein and Gerard (1934) expressed their results according to the van't Hoff-Arrhenius equation and thought that the critical thermal increments (μ) might indicate the nature of the oxidative processes of the unfertilized and fertilized egg. This view was criticized by Korr (1937), who stressed that biological scattering and the narrow temperature limits within which respiration can be measured make the graphical evaluation of μ-values uncertain. Moreover, biological processes are governed by enzyme reactions. Such reactions have repeatedly been found not to give constant thermal increments.

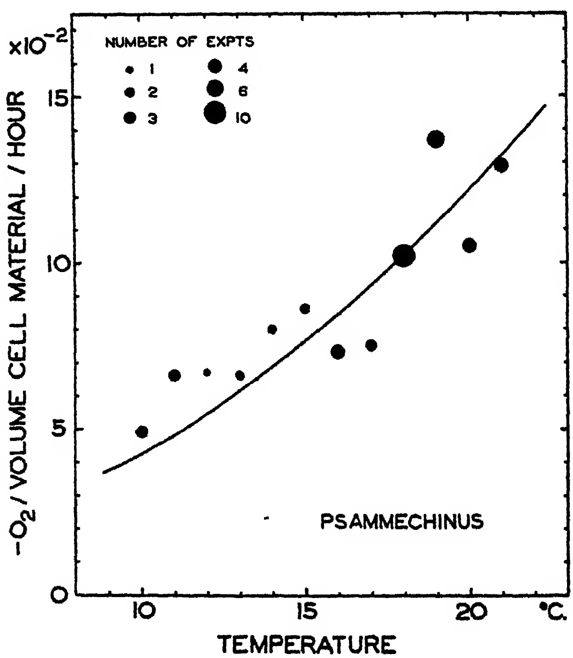

FIGURE 2. Oxygen consumption at different temperatures of unfertilized *Psammechinus miliaris* eggs.

Each dot is the mean value for the temperature in question. Diameter of dot indicates number of experiments. A standard curve according to Krogh (1914) is drawn in, passing through the value of 18° C.

The mass plot, recommended by Korr (1937), is difficult to interpret owing to the extent of the biological scattering. The mean of the values at each temperature gives a better representation. Figure 2 is plotted in this manner.

The figure shows that the standard curve of Krogh (1914) describes the obtained results quite well. This curve was originally obtained in basal metabolism experiments and found to be valid for a number of vertebrates. It was recently extended by Zeuthen (1947) to hold even for the respiration of a number of minute, chiefly marine invertebrate organisms. Thus Krogh's standard curve better describes the response in respiration on increase of temperature than does the van't Hoff-Arrhenius equation.

Formula (1)

For the temperature range of the present investigation the curve indicates that the relative temperature increment is very closely proportional to the relative increment in respiration. Thus it may be expressed by $(R_1/R_2)\,(t_2/t_1) = \text{const.}$, where R_1 and R_2 are the rates of respiration at the temperatures t_1 and t_2. The constant is dependent on the chosen difference between t_1 and t_2. For $t_1 - t_2 = 2°$ C., it is $= 0.93$.

In Figure 3 the results of this and previous investigations are given on a relative scale. The curve according to Formula (1) represents the material of this investigation. With this curve coincide the values of Tyler and Humason (1937) on the respiration of Strongylocentrotus eggs. On the other hand the results with Arbacia differ markedly from those on Psammechinus or Strongylocentrotus.

The temperature characteristics of the unfertilized sea-urchin egg thus represent two distinct classes: (1) the Arbacia type with high Q_{10} values, and (2) the Strongylocentrotus-Psammechinus type with Q_{10} values in close concordance with the standard curve of Krogh.

The temperature coefficients of the fertilized Psammechinus egg differ in no way from those found by previous authors for fertilized eggs of other sea-urchin species. Thus in the range 12–20° C. a $Q_{10} \sim 2\text{–}2.5$ was found.

A comparison of the Q_{10} values found in different investigations further stresses that there are two classes in respect to temperature characteristics of respiration of the unfertilized eggs (see Table II). Strongylocentrotus and Psammechinus have for both fertilized and unfertilized eggs a Q_{10} value at room temperature of approximately 2.5. The fertilized Arbacia egg shows the same value, but the value of the unfertilized egg is higher. The unfertilized egg of Paracentrotus has, at lower temperatures, a tendency in the same direction as the Arbacia egg.

Apparently Q_{10} as in most biological processes is higher at lower temperatures. The results of Tyler and Humason (1937) and of Korr (1937) seem, however, to contradict this conclusion, but the aberrations are probably to be attributed to experimental circumstances. (Lucké and co-workers, 1931, found that the Q_{10} of the permeability of the Arbacia egg to water increased with temperature.)

In the sea-urchin egg the rate of respiration is increased greatly by fertilization. This higher respiration is suppressed by cyanide, CO and other poisons of cytochrome oxidase. The oxidase in operation is an iron porphyrin, but probably not fully identical with the usual cytochrome oxidase. The respiration of the unfertilized egg was found by Runnström (1930) and Korr (1937) to be comparatively

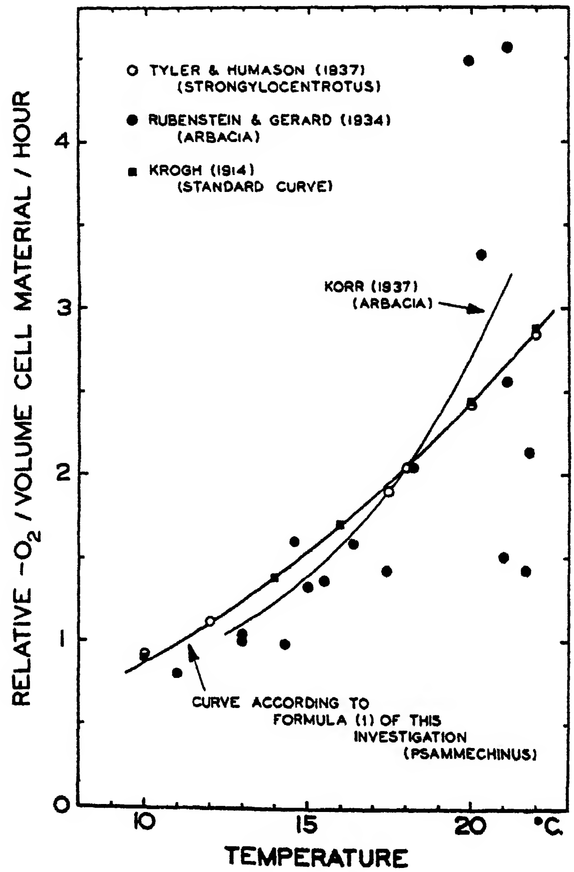

FIGURE 3. Comparison between different authors' results concerning temperature dependency of oxygen consumption in unfertilized sea-urchin eggs.

Rates of oxygen consumption given on a relative scale, putting the values at 18° C. alike. The curve for Korr's results is recalculated from this author's Figure 8.

slightly affected by said oxidase poisons. Both oxidase and dehydrogenases can be brought to work as effectively in the unfertilized egg as in the fertilized one. This permits the conclusion that neither the oxidase nor the dehydrogenases are the limiting factors in the respiration of the unfertilized egg (Runnström, 1935, 1942; cf., however, Ballentine, 1940). Runnström thinks that the difference in respiration rate between unfertilized and fertilized eggs is caused in the unfertilized egg by a lack in substrate saturation of the oxidase. Korr interprets the difference as being dependent on a factor in the oxidase part of the carrier chain, inactive or held apart in the unfertilized egg, but put into operation on fertilization. He thinks that this link might be cytochrome c and furthermore that the unfertilized egg respires over an autoxidizable, non-ferrous carrier. For a fuller review see Needham (1942) and Borei (1948a).

TABLE II

Q_{10} values of oxygen consumption of sea-urchin eggs

Author	Unfertilized eggs	Fertilized eggs
Loeb and Wasteneys (1911) †	(*Arbacia punctulata* 5–25° C. Q_{10}=1.31)§	*Arbacia punctulata* (round 1st mitosis) 3–27° C. Q_{10}~2–2.5
Rubenstein and Gerard (1934) *	*Arbacia punctulata* 13–30° C. Q_{10}=4.1	*Arbacia punctulata* (up to 5 hours after fert.?) 13–30° C. Q_{10}=1.8
Korr (1937) *	*Arbacia punctulata* 13–23° C. Q_{10}=3.7 18–28 4.5	*Arbacia punctulata* (up to 5 hours after fert.?) 13–23° C. Q_{10}=2.2, 2.8 18–28 2.5, 3.1
Ephrussi (1933) *	*Paracentrotus lividus* 10.4–22.4° C. Q_{10}=3.85 12.9–22.4 3.28 14.9–22.4 2.18 16.75–22.4 1.88	*Paracentrotus lividus* (gastrulae) 10.4–22.4° C. Q_{10}=2.36 12.9–22.4 2.57 14.9–22.4 2.32 16.75–22.4 1.88
Tyler (1936) *		*Strongylocentrotus purpuratus* (round 1st mitosis) 7.5–20° C. Q_{10}=2.54 10 –20 2.30 15 –20 1.85 (25th–26th hour after fert.) 15 –20° C. Q_{10}=1.88
Tyler and Humason (1937) *	*Strongylocentrotus purpuratus* 7.5–17° C. Q_{10}=2.67 8 –18 2.58 10 –20 2.63 12 –22 2.54 5 –20 2.35	*Strongylocentrotus purpuratus* (a few hours after fert.) 7.5–17° C. Q_{10}=2.79 8 –18 2.69 10 –20 2.26 12 –22 2.33 5 –20 2.62
This investigation ‡	*Psammechinus miliaris* 10–12° C. Q_{10}=3.52 12–14 3.07 14–16 2.81 16–18 2.58 18–20 2.40 20–22 2.29	*Psammechinus miliaris* (a few hours after fert.) 12–20° C. Q_{10}~2–2.5

† Winkler technique.
* Warburg experiments.
‡ Diver technique.
§ Only a single experiment.

Recent investigations on Arbacia eggs (Robbie, 1946) show that the respiration of the unfertilized egg can also be completely abolished by cyanide. A cyanide-stable respiration, catalyzed by formed CN-compounds, develops, however, after the initial inhibition. These findings confirm Lindahl's (1939, 1940 and 1941) results, and are in full concordance with his opinion that the cyanide-resistant respiration develops under the influence of the cyanide and that it has nothing to do with normal respiration. Robbie's findings show that the respiration of the unfertilized egg must proceed over iron porphyrins in the same manner as does that of the fertilized egg. Runnström (1930) had previously expressed the same opinion.

Robbie's results show conclusively that the respiration is of principally the same type both before and after fertilization. Thus it is understandable that temperature characteristics of respiration are found, as in the Psammechinus and Strongylocentrotus cases, that are the same before and after fertilization. On the other hand it is harder to understand how Q_{10} can differ so widely before and after fertilization as it does in Arbacia. It may be that the respiratory system operating in the unfertilized Arbacia egg is somehow unlike that of the just mentioned species. Any fundamental respiration pattern differences between the two classes of unfertilized sea-urchin eggs have, however, not been found.

Ballentine (1940) thinks that the oxidation in the sea-urchin egg of dimethyl-p-phenylenediamine requires cytochrome c as a mediator to the echinoderm oxidase. If so, cytochrome c must already be available and ready to function in the unfertilized egg, since Runnström (1932) and Örström (1932) found that dimethyl-p-phenylenediamine was oxidized at the same rate in unfertilized and fertilized eggs. Borei and Renvall (1949) could, however, not find that cytochrome c is essential for the cellular oxidation of dimethyl-p-phenylenediamine. Furthermore, hydroquinone, which can be oxidized by cytochrome oxidase (Keilin and Hartree, 1938) or echinoderm oxidase (Krahl and co-workers, 1941; Borei, 1945, Chapter IV: C1) only in the presence of a suitable mediator, has been shown by Runnström (1932) to be utilized in the unfertilized egg at a lower rate than in the fertilized one. This fact could possibly be taken as an indication that the carrier oxidized by the echinoderm oxidase has a lower concentration (or is less active or accessible) in the unfertilized egg than in the fertilized.

Many authors have doubted the existence of cytochrome c in the egg, as it has never been possible to find the cytochrome bands (cf. Krahl and co-workers, 1941). Thus Korr (1939), who originally (1937) thought that cytochrome c was released at fertilization, and Ballentine (1940) suppose that some other link, situated nearer the substrate, is put into operation at fertilization. It is, however, not unlikely that cytochrome c may have a sufficient carrier capacity in the sea-urchin egg, and yet not have a concentration high enough to permit spectroscopic detection. It may be pointed out (a) that the sea-urchin egg has a comparatively low Q_{O_2}, and (b) that cytochrome c is far more catalytically effective when attached to the proper intracellular protein particles than when working in solution. It may also be that another carrier has the same function in respect to echinoderm oxidase as has cytochrome c to cytochrome oxidase. (Concerning the differences between echinoderm oxidase and cytochrome oxidase, cf. Borei, 1945, and Krahl and co-workers, 1941).

The surplus in respiration induced by fertilization is either merely an addition to the respiration of the unfertilized egg, or caused by the fact that the induced respira-

tion might compete with and depress the latter. The smooth increase in post-fertilization respiration from the level of the unfertilized egg in Asterias speaks in favor of the addition possibility (cf. Borei, 1948a and Borei and Lybing, 1949), provided the respiratory mechanisms in eggs of starfishes and sea-urchins may be freely compared. The facts concerning temperature characteristics of oxygen consumption rates revealed in Arbacia, do not in themselves distinguish between the two possibilities, nor do any facts gained in this investigation concerning Psammechinus material. Concerning the declining pre-fertilization respiration see, however, Borei (1949), where it is shown that this respiration part does not influence post-fertilization respiration.

Parallels have been drawn between the respiration of the unfertilized sea-urchin egg and the diapause egg of the grasshopper. In respect to temperature characteristics, the grasshopper diapause egg has a very low Q_{10} in comparison with that of the active stages (Bodine and Evans, 1932), which is in contrast to the state in the sea-urchin egg. Too much stress may thus not be laid on such comparisons.

Summary

With Cartesian diver micro-respiration technique the temperature characteristics of the respiration of *Psammechinus miliaris* eggs were investigated:

1. Between 10 and 21° C. the gradual rise in oxygen consumption rate of the unfertilized egg is best represented by Krogh's standard curve. Q_{10} at 18° C. is around 2.5, at 10° C. around 3.5.
2. Fertilized eggs have the same temperature characteristics as unfertilized ones.
3. In respect to temperature characteristics just before and just after fertilization, two classes are distinguishable among the sea-urchins: The Strongylocentrotus-Psammechinus group with equal Q_{10} values before and after fertilization, and the Arbacia group with higher Q_{10} values before fertilization.
4. The significance of temperature characteristics for the biochemical processes involved in respiration of eggs before and after fertilization is discussed.

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INDEPENDENCE OF POST-FERTILIZATION RESPIRATION IN THE SEA-URCHIN EGG FROM THE LEVEL OF RESPIRATION BEFORE FERTILIZATION

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INTRODUCTION

The unfertilized sea-urchin egg just removed from the ovary respires at an oxygen consumption rate comparable with that of the newly fertilized egg (Borei, 1948a). After a few hours the respiration gradually drops, however, to a low, fairly constant level. This level represents the low respiration value of the unfertilized sea-urchin egg previously recorded in the literature. From this level there is a sudden jump at fertilization to the respiration rate of the newly fertilized egg. In Psammechinus the rate of the latter is usually three to four times that of the unfertilized egg at the constant level. For further details see Borei (1948a).

Now the question arises whether the increase in respiration after fertilization is merely an addition to the pre-fertilization respiration, or whether it competes with the existing respiration, eventually suppressing it completely.

In view of the concept (Runnström, 1930, Robbie, 1946) that the oxidase system in operation in the egg is the same before and after fertilization, and if parallels to events in the Asterias egg are considered (Borei and Lybing, 1949), the addition possibility is not unlikely. The difficulties arising in trying to interpret in this manner results from cyanide-inhibition experiments on fertilized eggs (Korr, 1937) could be avoided if Lindahl's (1940) findings are taken into account. He states that the cyanide-stable respiration arises under the influence of the inhibitor, and his view is strongly supported by Robbie's (1946) experiments.

On the other hand, Ballentine's (1940) concept that at fertilization a link in the dehydrogenase part of the oxidative chain is introduced, thus inducing an augmented respiration, offers a possibility that post-fertilization respiration, competing with that of pre-fertilization, may successfully suppress the latter, in spite of the fact (Robbie, 1946) that the same oxidase system is in operation both before and after fertilization. Korr, who first (1937) favored the view that cytochrome c is the link put into operation at fertilization, later (1939) abandons this and also turns to a concept that new ultimate substrate is released from precursors.

Runnström's (1930, 1932 and 1935) view that the oxidase is unsaturated with its substrate in the unfertilized egg, is equally consistent with both possibilities.

Borei and Lybing (1949) find that results from experiments on the temperature characteristics of sea-urchin eggs before and after fertilization can not decide between the two possibilities. It was stated by them that all facts at present known about sea-urchin egg respiration still leave the question open: It is just as possible to assume a simple addition as to believe in a competition.

It was thought that measurements of post-fertilization respiration in such experiments, where the eggs were fertilized at times corresponding to different points

on the decreasing respiration curve of the unfertilized egg, would help to elucidate the matter. If the rate of oxygen consumption before fertilization influenced the respiration of the fertilized egg, the addition possibility would have to be strongly considered. If not, one would be inclined to think that post-fertilization respiration is, as a whole, different from that before fertilization.

For a fuller discussion of previous literature on the biochemical aspects concerning sea-urchin egg respiration changes at fertilization, see Borei and Lybing (1949).

Material and Methods

The egg respiration of *Psammechinus miliaris* has recently (Borei, 1948a, Borei and Lybing, 1949) been studied in some detail. This species (phenotype S) was therefore chosen also in this investigation. For particulars concerning the material see Borei (1948a), Chapters 2.1, 3.111 and 3.21.

In order to make measurements possible on several lots of eggs taken from the same female and fertilized at subsequent times from the moment of removal from the ovary, one must use a method on the µl. scale. Thus Cartesian diver micro-respiration technique was employed. Concerning technical points see Borei (1948a), Chapters 2.2 and 2.3. Diver charge Type I (Borei, 1948b) was used throughout.

All experiments were performed at 18° C. After completed diver measurements, the cells were taken out of the divers and observed as to condition and fertilizability. The only experiments accepted were those in which these controls turned out satisfactorily.

In the actual runs, the procedure was as follows: Eggs were removed from the ovary, a lot was immediately fertilized, then a diver was charged with unfertilized eggs and these were brought to measurement as rapidly as possible. (The first respiration values could thus be obtained ~20 mins. after egg removal from ovary) This control diver was then continuously followed during all subsequent measurements on fertilized eggs from the same animal. From the first fertilized lot a diver was now charged. Subsequently new lots were fertilized and corresponding divers charged. In the experiments with fertilized eggs, the respiration rate at 120 minutes after fertilization, where the exponentially increasing respiration curve is still rather flat (see Borei, 1948a, Figure 3), was estimated and used for comparison. For unfertilized eggs a number of about 100, and for fertilized about 50, were found to be most suitable for charging the divers, which were of approximately 7 µl. capacity.

Results and Interpretations

The results from experiments on eggs from three females are represented in Figure 1.

It is obvious that respiration after fertilization reaches exactly the same level, irrespective of the height of the prevailing respiration at the moment of fertilization. It is of no importance whether the fertilization sets in at a very early moment, when the egg has just been removed from the ovary and accordingly respires at a very high rate, or whether it is effected a very long time after the removal, when the egg has alrady reached a fairly constant, low-rate respiration. In both cases the post-fertilization respiration will be the same.

In this connection it must be pointed out that the respiration of the fertilized egg in this investigation has been followed until more than nine hours after removal of the eggs from the ovary. This is considerably longer than in the cases reported by Borei (1948a), where the measurements were discontinued after about six hours. In no case, however, has there been observed any rise in the respiration rate at the end of the experiments, as might have been expected in consideration of the early findings of Warburg (1914) and Runnström (1928). Goldforb (referred to in Gerard and Rubenstein, 1934) found that the increase begins about five hours after shedding, and Tyler and Humason (1937) report a steady increase over the

whole measurement period. Runnström (1928) thinks that the increase is an indication of the "aging" of the egg (cf., however, Borei, 1948a, Chapter 3.113, who finds that over-ripe eggs have lower respiration than ripe ones and still lower than under-ripe ones). Tyler, Ricci and Horowitz (1938) found that the increase is avoided if the experiments are carried out under sterile conditions. It must be stated that all previous investigators have worked with Warburg technique. It might be that the dense packing of the eggs and the shaking conditions in this procedure support the growth of bacteria, whereas the Cartesian diver technique is more favorable in this respect. However, in this investigation no particular measures have been taken against bacterial contamination.

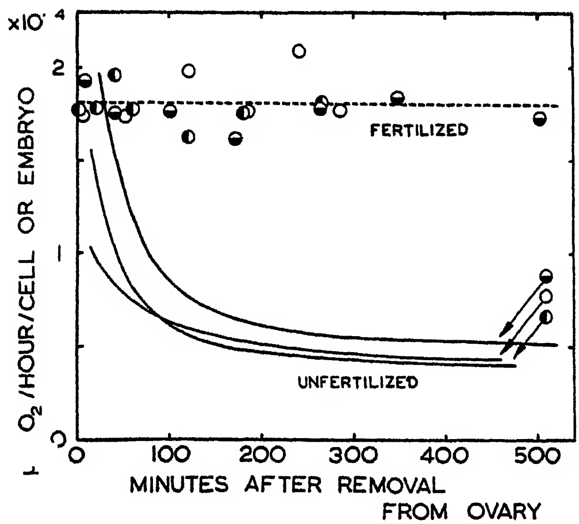

FIGURE 1. Oxygen consumption of unfertilized eggs and of fertilized eggs, fertilized at different times after the eggs' removal from the ovary. *Psammechinus miliaris.*

Each dot represents the respiration rate at 120 mins. after fertilization and is marked at the time of fertilization. ○, ◒ and ◐ stand for the three females used. Temperature 18° C. Oxygen consumption rate of unfertilized eggs at 230 mins. after removal from ovary: ○ = 0.50, ◒ = 0.58 and ◐ = 0.47×10^{-4} μl./cell and hour (mean value found by Borei 1948a = 0.53); mean value of rates of fertilized eggs: ○ = 1.85, ◒ = 1.78 and ◐ = 1.79×10^{-4} μl./embryo and hour (Borei, 1948a = 1.84). For the respiration of fertilized eggs, the best fit according to the method of least squares is indicated by a dotted line.

Borei (1948a, Chapter 3.21) compares the quotient between respiration after fertilization and that before, and finds that the values from different sea-urchin species vary considerably, and moreover that for one and the same species, greatly differing values are reported. So for example for *Arbacia punctulata* quotients from 2.6 to 5.3 have been given. For Psammechinus the values 3.6 and 5.7 are recorded. On the other hand, if the conditions of measurement are well defined as to time from fertilization and from removal from ovary respectively, the quotient will become fairly constant, as the results of Borei (1948a, Chapters 3.112.2 and 3.21) show. Considering the possible influence of values of pre-fertilization respiration on the quotient, obtained at different points on the declining egg respiration curve, Borei (1948a) thinks that quotient values given in the literature are of minor importance for quantitative considerations concerning respiration changes at fertili-

zation, but merely show that the oxygen consumption of the unfertilized egg some few hours after its removal from the ovary is considerably lower than that of the fertilized egg some few hours after fertilization. The present investigation supports this concept thoroughly. If the quotients from the experiments in Figure 1 are recorded (Table I), it is clearly seen that the quotient value will become smaller and smaller the closer the time of fertilization lies to that of egg removal. It would rather seem that values < 1.0 could be obtained in the earliest experiments, i.e. that the respiration of the just removed egg is actually higher than that of the fertilized egg during the first hours of development (cf. Borei, 1948a).

TABLE I

Quotients between oxygen consumption rate 120 mins. after fertilization and oxygen consumption rate of unfertilized eggs at the moment of fertilization. Psammechinus miliaris

Same experiments and denotations as in Figure 1. Value given by Borei (1948a) for 230 mins. after egg removal from ovary = 3.6. In quotients marked * respiration values of unfertilized eggs are obtained graphically from extrapolated curves.

○		◒		◐	
Mins. after egg removal	Quotient	Mins. after egg removal	Quotient	Mins. after egg removal	Quotient
6	<1.6*	8	<1.0*	2	<1.0*
52	2.3	40	1.2	21	1.2
120	3.3	100	2.2	41	2.5
185	3.5	170	2.5	60	2.8
241	4.3	264	3.2	120	3.1
286	3.8	349	3.5	179	3.6
		502	3.4	265	3.9

It would appear from the presented data, as the value of the pre-fertilization respiration rate seems to be of no importance for the oxygen consumption rate after fertilization, either that pre-fertilization respiration constitutes no integral part of the respiration after fertilization, or that fertilization brings about a release from inhibiting factors active on respiration in the unfertilized egg. As the oxidase system is probably the same both before and after fertilization (Runnström, 1930, Robbie, 1946; see also Introduction of the present paper), it is reasonable to suppose that changes occur at fertilization in those parts of the system which are situated between the oxidase and the dehydrogenases. The dehydrogenases themselves, however, are not likely to be affected. (Dimethyl-p-phenylenediamine experiments by Runnström, 1930 and 1932, Örström, 1932, Borei and Renvall, 1949; hydroquinone, Runnström, 1930; pyocyanine, Runnström, 1935; methylene blue, Runnström, 1930; cf., however, Ballentine, 1940, who claims that the dehydrogenases are not capable of maximum activity in the unfertilized egg.) (Cf. Korr, 1939: "release of substrate from precursors.")

From experiments on the respiratory quotient of pre- and post-fertilization respiration in the sea-urchin egg, it appears very probable that different substrates are utilized before and after fertilization. These changes in RQ upon fertilization,

which support the view that post-fertilization respiration competes with that of pre-fertilization, eventually suppressing it more or less completely, are seen from Table II.

It must, however, be kept in mind that a constant respiration part is assumed for the unfertilized egg (Borei, 1948a, Chapter 3.112.1) as well as a decreasing part. The above-presented data do not indicate whether even the constant respiration part is abolished at fertilization. It is still an open question whether this part of pre-fertilization respiration survives fertilization or not.

TABLE II

RQ of sea-urchin egg respiration before and after fertilization

Time in relation to fertilization	RQ	Species	Author
Before*	1–1.2	*Psammechinus*	Borei (1934)
0–30 mins. after	0.84		Laser and Rothschild (1939)
30–40 mins. after 40–50 mins. after	0.78 0.64		Borei (1934)
35–50 mins. after	0.66		Laser and Rothschild (1939)
1–2 hr. after 7–8 hr. after	0.73 0.85	*Paracentrotus*	Öhman (1940)
2-cell stage-hatching	0.8		Ephrussi (1933)

* Ashbel (1930) finds the value 1.06 before fertilization (Arbacia).

SUMMARY

Using Cartesian diver micro-respiration technique, it was found that in *Psammechinus miliaris* the rate of respiration of the newly fertilized egg is independent of the rate of respiration of the unfertilized egg at the moment of fertilization.

The quotient (respiration after fertilization)/(respiration before fertilization) was found to decrease considerably (probably even to values < 1.0) if the time interval between egg removal from the ovary and fertilization was diminished. If the decreasing part of pre-fertilization respiration is given time to disappear before fertilization, the quotient lies between 3 and 4.

It is thought possible that the decreasing respiration part of the unfertilized egg is abolished upon fertilization, due to probable changes in the function of members of the oxidizing system, situated between the oxidase and the dehydrogenases. It cannot be decided from the experiments whether the constant respiration part of the unfertilized egg still participates in the respiration of the fertilized egg or not.

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STUDIES ON THE THERMAL DEATH OF HYALELLA AZTECA SAUSSURE [1]

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Introduction

Much work has been done in the past half century concerning the effects of temperature on the survival of organisms. However, little has been done in that respect with the Crustacea, particularly the fresh water forms.

A study was made from June 16, 1947, to August 15, 1947, concerning the effects of temperature on the survival and death rates of the amphipod *Hyalella azteca* Saussure (*Hyalella knickerbockeri* Bate, *Hyalella dentata* Smith). This amphipod is abundant in the waters of Little Miller's Bay in West Okoboji Lake, Dickinson County, Iowa, at the site of the Iowa Lakeside Laboratory, where the investigation was conducted.

Historical Survey

Geisler (1944) suggests that rate of development in *Hyalella azteca* is directly related to the temperature, but does not record the effects of higher temperatures. For marine copepods and decapods, Huntsman and Sparks (1924) report heat death at temperatures between 22° and 33° C. when the animals were exposed to temperatures rising at the average rate of 0.2° C. per minute. Brown (1928) reported a temperature characteristic, μ, of 187,000 calories from 35° to 41° C. for thermal death in *Daphnia magna*.

Materials and Methods

Numerous Hyalella were secured daily by placing fresh masses of the green alga, *Cladophora fracta*, in which they feed, in a pail of lake water. Amphipods which came to the surface of the water were transferred to a stock tank of fresh lake water at 20° to 22° C. by means of a tea strainer. This lake water was partially changed daily and replaced weekly, and was used in all experiments. The temperature of the tank water was about the same as that of the natural water where the animals were taken.

The water baths used in the experiments were five-gallon containers heated electrically and controlled manually to ± 0.2° C.

[1] Undertaken in partial fulfillment of the degree of Master of Science at the State University of Iowa. The author wishes to express his gratitude for helpful criticism of the initial and final drafts of the paper on the part of Dr. Theodore Louis Jahn of the University of California, Los Angeles, formerly of the University of Iowa, and Dr. Robert L. King of the University of Iowa, at whose suggestion the problem was begun.

[2] On leave of absence for research and study at the University of California, Los Angeles.

In short exposures several hundred Hyalella were dipped from the stock tank with a tea strainer, over which a muslin square was then fastened and held taut with a rubber band. The strainer was immersed and oscillated in the water bath for the desired period. Then the Hyalella and the strainer were transferred quickly to enamelled basins of fresh lake water at 20° to 22° C.

In exposures longer than thirty seconds small tin cans with tops and bottoms removed were employed. Muslin squares fastened over the open ends kept the amphipods confined, but exposed to the water. A number of these tins were placed in the water bath and removed as desired.

The water bath was stirred between short exposures, and during exposures longer than thirty seconds was continually aerated with compressed air. The tins containing the Hyalella were placed on a wire platform as near the center of the water bath as possible, and at least six inches away from the source of heat.

Exposed animals were allowed to remain in recovery basins four or more hours before counting. Counting was usually done six to eight hours after exposure. Check counts showed that injured animals which were alive four hours after exposure were always dead after sixteen hours. Therefore, injured animals were counted as dead. Control animals remained alive, except for rare exceptions, after twenty-four hours in the basins.

Water from each basin was individually strained through muslin to collect the the amphipods for counting. The muslin was spread flat on a moist, concrete slab for counting under a sixty-watt electric bulb. Because of negative tropism to light (Phipps, 1915), the living amphipods crawled toward the periphery. A few cubic centimeters of water dropped at the center of the muslin hastened the outward movement. After thus separating the living and dead, the living were counted first. Those evidencing injury or feebleness when stimulated with water or the points of a pair of tweezers were counted as dead.

Mature specimens were separated into two groups; so also were immature specimens. Those longer than 7 mm. were considered very large adults; those from 3½ to 7 mm. as large adults; those from 2½ to 3 mm. as medium-sized adolescents; and those still less as small and juvenile (Geisler, 1944). A separate count was taken according to sexes for each of the adult groups on the basis of salient characteristics (Geisler, 1944) as seen through a dissecting scope.

Time intervals were determined with a watch calibrated in fifths of a second.

Experimental Results

Survival in constant temperature baths

Constant temperatures used included one degree intervals C. from 38° to 50°. Tests were also run at 36.5°, 35° and 33°.

Thermal death times and temperatures here shown are those at which fifty percent of the organisms survived.

At 50°, less than one second was sufficient time to cause thermal death. At lower temperatures the time increased gradually so that at 40° an exposure of 75 seconds was needed. Below 40° the increase in time required to kill was very sharp, so that at 33° more than eleven hours (39,600 seconds) was necessary (Table 1).

Size and survival in constant temperature baths

Age and size are directly correlated in Hyalella; the larger the animal, the older it is (Geisler, 1944). Large adults showed the greatest resistance, usually higher than the average figure for the total of all simultaneously exposed. Very large adults varied in their resistance, but their rate of survival usually approached the average. Medium adolescents showed still less resistance; and small juveniles were least resistant (Table 1).

TABLE 1

Heat death for Hyalella azteca in constant temperature baths

Degrees C.	Time exposed in seconds	Per cent survivors					Total number animals exposed
		Total animals exposed	Very large adults	Large adults	Medium adolescents	Small juveniles	
50.0	1	50.8	52.1	62.5	45.6	43.3	240
49.0	4	51.2	64.8	59.4	47.5	44.4	416
48.0	7	50.3	48.7	51.8	52.0	47.6	320
47.0	8	50.8	58.3	57.2	48.2	46.6	114
46.0	12	49.3	31.7	51.9	54.3	47 1	162
45.0	14	47.1	52.2	65.4	36.3	43.6	318
44.0	15	55.0	40.1	56.2	56.7	49.4	623
43.0	24	49.5	58.5	63.6	43.0	43.3	656
42.0	39	49.9	52.8	56.4	49.1	37.9	204
41.0	60	47.6	33.6	67.6	51.3	46.1	432
40.0	75	49.6	49.9	68.8	47.6	46.8	626
39.0	135	49.3	49.3	58.4	44.6	48.4	636
38.0	240	48.9	49.2	55.8	45.0	46.9	547
36.5	1,800	49.9	44.8	52.9	51.1	53.6	210
35.0	6,300	51.8	50.2	56.2	46.1	46.8	583
33.0	39,600	53.7	51.2	51.9	51.5	48.2	146

Sex and survival in constant temperature baths

No valid evidence was found to indicate that sex affects resistance to heat in constant temperature baths. For all adult specimens, male survivors outnumbered females in sixteen out of thirty-two cases.

Survival in rising temperature baths

Using the same equipment as that employed for the constant temperature baths, Hyalella were exposed to four average rates of temperature rise, beginning at 20° to 22° C. Rates of rise were: 0.375° per minute; 0.261° per minute; 0.150° per minute; and 0.036° per minute. At lower temperatures the rate of rise per degree C. was more rapid than at higher temperatures. The rates of rise are here expressed as average rates, in order to make them comparable to those of other investigators who previously encountered the same difficulty (Huntsman and Sparks, 1924). Within the rates of rise investigated, thermal death did not occur below 39° C. and always was found above 41° C. (Table 2).

TABLE 2

Heat death in Hyalella azteca in rising temperature baths

Rate of rise in degrees C per minute from 20° to 22° as a base	Degrees C.	Total animals exposed	Per cent survivors				
			Total animals	Very large adults	Large adults	Medium adolescents	Small juveniles
0.375	37.0	178	89.4	94.6	92.6	88.8	74.1
	38.0	212	84.4	85.7	77.8	75.8	75.0
	39.0	189	87.8	93.7	88.8	82.7	78.3
	40.0	201	51.7	52.7	50.2	50.0	51.8
	41.0	170	60.2	69.7	54.8	52.6	50.0
	42.0 to 45.0	#200@					
0.261	37.0	462	85.9	95.5	96.3	89.1	83.1
	38.0	470	81.1	91.1	83.7	77.2	76.7
	39.0	456	83.9	93.1	83.1	83.7	78.6
	40.0	534	68.1	81.0	79.9	63.1	64.6
	41.0	340	27.6	31.2	23.6	26.5	29.0
	42.0 to 45.0	#400@					
0.150	37.0	421	93.5	96.4	95.8	86.5	87.5
	38.0	492	82.9	92.3	85.8	76.8	77.1
	39.0	493	77.4	88.6	76.9	75.4	66.6
	40.0	430	70.7	75.0	73.0	65.3	65.5
	41.0 to 45.0	#400@					
0.036	36.0	464	94.8	97.6	96.0	89.6	91.8
	37.0	306	86.9	94.6	95.6	82.5	78.7
	38.0	493	65.9	73.5	81.5	59.8	55.9
	39.0	194	29.3	12.7	39.0	42.0	25.6
	40.0 to 45.0	#350@					

* Average rate of rise.

Approximate number at each degree of temperature within the bracketed limits; no accurate count taken since all were dead.

Size and resistance to rising temperatures

Very large adults demonstrated the highest resistance in rising temperature baths, except at the slowest rate of rise. Large adults, medium adolescents, and small juveniles showed, respectively, less resistance (Table 2).

Sex and resistance to rising temperatures

No evidence was found that sex causes any variance in resistance to rising tem-

peratures. Male adult survivors in some trials outnumbered females, and vice versa, but never in significant numbers.

Adjustment to rising temperatures of water

A temporary adjustment to rising temperatures was noted. For example, organisms plunged into and continuously exposed to a pre-heated constant temperature bath at 38° C. reached thermal death in four minutes. Within the rates of rise investigated, thermal death was not found to occur at 38°, 87.8 per cent still surviving at that temperature at the fastest rate of rise, and 65.9 per cent surviving at the slowest rate of rise (Table 2).

Temperature coefficients for thermal death

Adaptations of the v'ant Hoff-Arrhenius equation are often used to express the rate of progress in biological reactions, although it is possible that such characteristics are more descriptive than analytically accurate.

Computation and comparison of Q_{10} for a number of temperature ranges within the full range investigated revealed that although death occurs more quickly at higher temperatures, the rate at which the lethal effect progresses decreases as the temperature increases. The decrease in Q_{10} was very marked for intervals below 40° C. Above that temperature there was a sharp break in the deceleration of the rate of progress and the Q_{10} variance was not so great (Table 3).

TABLE 3

Temperature characteristics for Hyalella azteca for thermal death in constant temperature baths

Temperature intervals in degrees C.	Q_{10}
33.0–35.0	9,768.00
35.0–40.0	7,056.00
38.0–43.0	100.00
40.0–45.0	28.91
43.0–48.0	11.75
45.0–50.0	196.00*

* Apparent divergence may be due to experimental inaccuracies.

SUMMARY

1. Thermal death occurs in *Hyalella azteca* at constant temperatures from 33° to 50° C. The time required to produce thermal death varies from more than eleven hours (39,600 seconds) at 33°, to less than one second at 50° C.

2. Comparison of Q_{10} values for narrow ranges within the broad range of temperatures investigated indicates a marked decrease of Q_{10} values at higher temperatures in spite of a more rapid lethal effect.

3. Thermal death occurred in rising temperatures, the slower the rate of rise, the lower the killing temperature, being not below 39° nor above 41° for the rates of rise investigated.

4. A temporary adjustment was found to occur to rising temperatures, delaying thermal death at a given temperature for some time past the period necessary to kill on immersion in the constant temperature bath at the given temperature.

5. Resistance to the effects of heat appears to be directly related to the size and age of the animal, the older and larger the animal the greater the resistance, except for the largest animals (which might have reached a state of senility).

6. Resistance to the effects of heat does not appear to be related to sex in *Hyalella azteca.*

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REGENERATION IN AN EARTHWORM, EISENIA FOETIDA (SAVIGNY) 1826. I. ANTERIOR REGENERATION

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These contributions present the results of an attempt to obtain for one particular species of earthworm complete characterization of regenerative capacity with reference to exact levels. In this part anterior regeneration, by posterior substrates only, is considered.

Summary of Previous Work

Information available as to the morphological nature and segmental constitution of anterior regenerates on posterior substrates is summarized, with certain reservations, in Table I.

In earlier work on *E. foetida,* as well as other species of earthworms, determination of morphological nature of regenerates seemed unnecessary. Later, Michel (1898, p. 283), recalling Bonnet's heteromorphic tails in aquatic Oligochaeta, suggested that two of his own anterior regenerates, as well as some of those of Joest and Rievel, were caudal. Although anterior heteromorphosis was definitely confirmed by Morgan (1899) no attempt was made then or since to clarify the situation, and in particular to determine the limits of homomorphic head and heteromorphic tail regeneration.

The consequent uncertainty as to morphological nature of regenerates at a considerable number of levels and even as to the levels (because of postregeneration estimation), as well as absence of data for numbers of levels and paucity at other levels, indicated the advisability of a systematic investigation of regeneration at each level from ½ posteriorly.

Materials and Methods

Material was first secured from a heap of decaying leaves, later from manure heaps. Worms were kept in moist filter paper or paper towelling until the gut was cleared. Individuals with any indication of damage by collecting, disease, abnormality, homoeosis or previous regeneration were rigorously rejected, and only those which were clitellate, or which had been clitellate when brought into the laboratory, were used. Animals were kept throughout at ordinary room temperature, which in winter probably was never above 68° F.

Anaesthesia was brought about in 0.2 per cent chloretone. Transections were made under a dissecting binocular microscope exactly across the animal on an intersegmental furrow.

After operation worms were placed in water until recovery from anaesthetic and were then transferred to filter paper, paper towelling, or cheesecloth. On several

TABLE I

Anterior regeneration in Eisenia foetida

Level	Regenerate: Cephalic, Number of segments: 2	3	4	5	6	7	?	Uncertain	Caudal	None	Comments	Author	Date	Page
1/2											See note (a)			
2/3	6											Morgan	1895	447
3/4	5	9										Morgan	1895	447
	4	1										Michel	1898	261
4/5	1	8	5									Morgan	1895	448
		1										Hescheler	1896	228
	3	10	1									Michel	1898	262
5/6		2	3								See note (b)	Morgan	1895	448
	1	8	5									Hescheler	1896	228–31
			1	1								Michel	1898	268
EL		5	5	1							See notes (c) (d)	Morgan	1895	453
6/7		2	3									Hescheler	1896	228
EL	1	4	6	1								Morgan	1895	453–6
7/8		1										Hescheler	1896	228
						1					See note (e)	Michel	1898	262
EL		1	9									Morgan	1895	453
8/9			1	3	1							Michel	1898	262
EL	1	3	5	1								Morgan	1895	455
9/10 EL	1	1		3								Morgan	1895	453–6
10/11			3					1			U="Imp"	Morgan	1895	451
			2	5	3							Carpenter	1948	625–6
EL					1							Morgan	1895	455
11/12			3	1								Michel	1898	263
EL			1	1				1			U="2 (or three very imperfect)"	Morgan	1895	456
12/13		1		2	1							Morgan	1895	451–6
	1	1		1				1			U:S=indistinct	Michel	1898	263
							2	0	0		100–0–0%	Dimon	1904	350
EL		1										Morgan	1895	456
13/14 EL		1										Morgan	1895	456
14/15				1								Morgan	1895	451
							10	0	0		100–0–0%	Dimon	1904	350

TABLE I—*Continued*

Level	Regenerate: Cephalic, Number of segments: 2	3	4	5	6	7	?	Uncertain	Caudal	None	Comments	Author	Date	Page
15/16 EL							22 3	2# 4	0 1?	 5	92–8–0% ?="possibly a new tail"	Dimon Morgan	1904 1902	350 579
16/17 EL							11	1# 2	0		92–8–0% 3–4 S, "not regenerated (mouth present)" (See note (f))	Dimon Morgan	1904 1895	350 455
17/18							12	1#	0		92–8–0%	Dimon	1904	350
18/19							26	5#	2		79–15–6%	Dimon	1904	350
19/20							 3	1 ?#	 ?		U="4 or 5 S" See note (g)	Morgan Dimon	1895 1904	451 350
20/21 EL									1		17 S See note (h)	Morgan	1899	409
?							4	7#	12		17–30 5–52 5% See note (g)	Dimon	1904	350
22/23								2			Very imperfect	Morgan	1895	452
23/24 EL								1 1			Very imperfect 3 or 4 S, imperfect	Morgan Morgan	1895 1895	452 456
24/25								2			See note (h)	Morgan	1897	574
EL 25/26							1		1		T=17 S See notes (h) and (i)	Morgan	1899	40
EL 30/31							1		1		H=7 or 8 S See note (h) T=21 S	Morgan	1899	408
EL 34/35				1								Morgan	1901	7, Fig. G
EL 35/36									1		(L 63 S) T=15+S See notes (h) and (i)	Morgan	1895	455
L 75 S									1		35 S	Morgan	1899	409
EL 50/51				1				 2	 14	 15	 T=5–25 S	Morgan Morgan	1901 1902	7, Fig. H 579

TABLE I—*Continued*

Level	Regenerate										Comments	Author	Date	Page
	Cephalic Number of segments							* Uncertain	Caudal	None				
	2	3	4	5	6	7	?	*						
L20S										5		Michel	1898	263
L10S										5		Michel	1898	263
L5S										5		Michel	1898	263
L12–7S										98		Morgan	1897	575–6

NOTES TO TABLE I

(a) "Attempts made to cut off 1 and 2 segments" (Morgan, 1895, p. 449). As a result of confusion re numbering of containers there was but one case in which it was thought "one segment must have been cut off" and that specimen could have been a posterior homoeotic.

(b) The last two specimens in Morgan's Table IV were homoeotic and are here excluded. The three specimens next above are assumed to have been normal. (Homoeotics are excluded here, as well as from author's operations, to obviate possibility of complications resulting from a previous regeneration and because gradients cannot be expected to be the same as in normal specimens.)

(c) Amputations, apparently as a result of operating without anaesthesia, were often diagonal (Morgan, 1895, p. 457, also Korschelt), or if transverse then at an intra- rather than intersegmental level. In each case a portion of a segment is treated as if a whole segment, i.e. if $10\frac{1}{4}$ or $10\frac{1}{2}$ segments were removed from the anterior end, the level of regeneration is still considered to be 10/11. In favor of this convention is Morgan's conclusion, after study of deliberately made very diagonal cuts, that simultaneous completion of missing parts of segments did not interfere with replacement of those metameres that had been completely amputated (1895, p. 457).

(d) EL estimated level. Level of amputation in many operations was estimated *after* regeneration and from one of the following landmarks (Morgan, 1895, pp. 450 and 452): (1) Position of vasa deferentia, i.e. location of male pores. Subject to variation of six segmental levels (Morgan, 1895, p. 403). (2) Location of seminal receptacles, apparently thought to be three pairs. *Eisenia foetida* has only two pairs of spermathecae but four pairs of seminal vesicles. These landmarks are doubtful. [If three pairs of spermathecae were present another species was involved, possibly *Dendrobaena octaedra* (Savigny) 1826 or *Allolobophora chlorotica* (Savigny) 1826, both of which are found in compost heaps and apparently have been confused with *foetida*.] (3) Location of clitellum. That may begin on any of segments xxiv–xxvii and end on xxxi–xxxiv, a variation of three to four levels. Pre-clitellar amputation was variously listed as at 20/21, 25/26, and postclitellar as at 25/26, 30/31, 35/36. (4) and (5) The middle and the end of the body, the former regarded as at 50/51 and the latter as the hundredth segment. Number of segments varies from 67–125.

Actual variations, when recognizable, were: for (3) of seven segmental levels, i.e. 19/20–26/27 and 31/32–38/39, for (4) and (5) to about 20 levels, 41/42–64/65, etc.

Postregeneration determination of level of amputation would probably render unlikely detection of reorganization of substrate segments. Such reorganization, in some species, could affect the determination by one to three segmental levels.

In certain of Morgan's cases it is not clear whether levels mentioned were determined or estimated.

(e) Number of segments of other regenerates at this level "très variable."

(f) The regenerate segments were "very irregular." The characterization "not regenerated

occasions when the supply of cloth had been exhausted, worms were kept in large crystallizing dishes in water just sufficient to cover the bottom and keep the animals moist. Although *E. foetida* appeared to do as well in water, in cool weather, as in moist cloth, the method is not recommended, for in later work several long series of operated animals were completely lost over night. To prevent accumulation of metabolic wastes, water or paper was changed (or cloth washed) daily, except on Sunday when the museum was closed.

Specimens were killed so as to insure uniform contraction and were then preserved in formalin.

The experiments were carried out mainly during a sabbatical leave in the States in 1926–27. Shortly before Japan entered the war, a summary of the results obtained was prepared and sent home from Burma. Original records, as well as specimens, were lost in the sack of Rangoon.

The author's thanks are extended to Prof. G. H. Parker for provision of laboratory facilities at Harvard University during the academic year 1926–27, to Prof. H. W. Rand for similar facilities in the U. S. Fish Commission building at Woods Hole during the summer of 1927, to Dr. Esther Carpenter for care of operated animals while the author was ill.

Nomenclature

In one and the same article, an author once used "posterior end" to refer to: a posterior regenerate regardless of size; an anterior regenerate (heteromorphic); the anal region of an adult worm; and long posterior portions of varying lengths up to a half or more of adult size. Similarly "anterior end" has had various meanings, including even that of tail (heteromorphic). Most confusing, however, has been a failure to distinguish adequately in discussions between the regeneration taking place at a single surface of amputation and that taking place at exactly the same level when there are two cut surfaces. In an attempt to avoid further complications, an effort has been made to restrict terms and phrases consistently to the meanings given herewith.

(mouth present)" may refer to an anally sculptured cicatrix. Such sculpturing may be preliminary to growth of a tail regenerate.

(g) Results of all operations behind 18/19 were lumped together. Mention was, however, made of three "B" heads at 19/20 which have also been listed above at that level.

(h) No data as to number of segments in 1897 regenerates (Morgan, pp. 573–574) and no clues to warrant guesses as to nature of regenerates.

(i) Results of 30 operations (Morgan, 1902, pp. 578–579) omitted because of uncertainty: (1) as to level of operation, said to have been "just behind the girdle (about the 25th segment)," i.e. either at 25/26 or 34/35; (2) as to nature of substrate, i.e. whether posterior or a two-surfaced fragment. Three months after operation, four specimens having died, the container had 36 specimens which were not examined for autotomy. *Nevertheless*, presence of one distinct new head and 14 doubtful regenerates, of which "probably more were heads than tails" may be of considerable importance.

Presence of extra worms in containers may have another explanation than autotomy. Just hatched juveniles are exceedingly difficult to find in either manure or soil. In absence of sterilization of the manure used for culture medium, there was time, during the months allowed for regeneration, for young to attain adult size. In this connection a belief that regenerates became indistinguishable from substrates is perhaps important (Morgan, 1895, p. 424).

Regenerates referred to this class by Dimon were not characterized in any way. * Some "doubtful" regenerates of other authors are also included here. Others, that appear also to be doubtful, have been included in part.

In place of regenerant and regenerate, which are easily confused, *substrate* and *regenerate* are used respectively, to designate the portion of the original worm on which the new growth is formed and the new growth thus formed at the cut surface. This is in continuation of previous practice (Gates, 1941).

Posterior substrate refers to any posterior portion of the body, regardless of size, extending forward from the anal region to a single anterior cut surface.

Healing after amputation may be *cicatricial* or *enteroparietal*. In the first, a cicatrix is formed across the cut surface, while in the second, cut edges of gut and body wall apparently heal together without definitely recognizable cicatricial tissue.

A regenerate with no externally recognizable differentiation is a *bud* (indeterminate). As indications of buccal or anal sculpturing become recognizable, further characterization as cephalic or caudal is possible. With appearance of metameric differentiation the regenerate is a head or a tail.

A considerable degree of deviation from normal structure may be possible in a regenerate without affecting its caudal or cephalic nature. Such variant regenerates are abnormal. A regenerate without cephalic or caudal characteristics, or with a mixture of cephalic and caudal characteristics, or with bifurcations, is a *monstrosity*. A growth without indication of caudal or cephalic nature is an indeterminate monstrosity.

A metamerically normal regenerate may be *equimeric, hypermeric*, or *hypomeric*, depending on whether it has the same number of segments, more than, or fewer than the excised portion.

Heteromorphosis indicates a more or less normal structure in a reversed or abnormal direction. A head at a posterior amputation and a tail at an anterior amputation is heteromorphic. Homomorphic distinguishes the head or tail in normal position or direction.

Levels are designated in two ways, by reference to the segments, as xxvi, and to the furrows bounding the segment as 25/26 and 26/27. The Roman numeral in lower case means the twenty-sixth segment beginning with the buccal as i; the prostomium of the Oligochaeta is not counted as a segment. The fractions refer to the intersegmental furrow at the anterior and posterior margin of segment xxvi, and make possible, with shorthand brevity, exact designation of level. Indication of level of amputation merely by reference to the segment, as "at the twenty-sixth segment," may be inadequate unless the context indicates which of the two possible levels, anterior or posterior, is involved. EL before the fraction means *estimated level*, the estimate usually that of the original author, otherwise made in accordance with his custom so far as is possible.

The anal region of the body forward to the first complete intersegmental furrow is not regarded as an ordinary metamere but for purposes of segmental enumeration is taken as one segment (see Gates, 1948). Posterior substrates of unknown location with reference to the antero-posterior axis are characterized by a designation such as L14S, in that case meaning the last fourteen segments.

Homoeosis, as ordinarily used in connection with earthworms, means: presence of an organ or pair of organs, or a series of organs, in a segment or series of segments, other than that, or those, in which usually or normally found. It refers primarily to individual variation within a species; secondarily, to phylogenetic variation, for a species or a genus may be homoeotic with reference to other species in the genus, or other genera in the family. In case of individual homoeosis, the dislocation may involve one or both organs of a pair in a segment. The former is asymmetrical homoeosis, the latter symmetrical.

Summary of Results

Healing at cuts in an anterior portion of the body was cicatricial, the cicatrix a low, flat-surfaced, circular disc without recognizable sculpturing. In some specimens no further development was recognizable. In others the cicatricial disc gradually was protruded as a small, rather conical bud at first apparently unmarked by any sculpturing. In several cases the growth of the bud was inhibited at that stage. In the remainder the distal portion became sculptured to indicate a prostomium and mouth. Intersegmental furrows, setae, and finally pigment usually became recognizable in that order.

Regenerates always remained distinguishable from substrates by differences in pigmentation, segment size, setal intervals, etc.

Several months' starvation resulted in reduction of size of substrates but no macroscopically recognizable reorganization was noted, either externally or internally, behind the level of amputation.

Reproductive organs were not found, in regenerate or (as result of reorganization) in substrate.

A. After a single cut

All substrates with cut surfaces at levels from 8/9 anteriorly, with one exception, regenerated. At each level behind 8/9 one or more of the substrates did not survive operation long enough to regenerate or else failed to regenerate if surviving. Highest percentages of failure to regenerate were in the region around 25/26. Further posteriorly, survival was better and percentages of successful regeneration higher. Results just mentioned were, however, minimal, as inhibited buds, rare monstrosities (indeterminate) and certain conditions to be considered later on were recorded as failures (to produce a more or less normal head or tail).

Head regenerates were obtained at levels 1/2–23/24 only. Equimeric heads were obtained at levels 1/2–8/9 inclusive. Three regenerates at 4/5 were hypermeric (+ 1). All head regenerates at levels 9/10–23/24 were hypomeric, the maximum number of segments obtained being six. In a later series of operations, E43, of three head regenerates at 8/9, one had five, another had six, and a third had nine and a half segments, the half segment wedge-shaped and on the right side (+ 1½).

Heteromorphic tail regenerates were obtained, once each at levels 20/21 and 23/24, and from 24/25 to 54/55. The largest number of setigerous segments differentiated in such heteromorphic tails was 25 at 40/41, the evidence available indicating increase in number of segments posteriorly to 40/41 and then a decrease.

At levels behind 54/55 no regeneration whatever, including even buds and monstrosities, was obtained though numbers of substrates were under observation three to four months.

B. After a previous regeneration

In attempts to test for the effects of previous regeneration on anterior regeneration, several series of operations were made of which the following are mentioned.

In series E41 posterior portions were removed at 34/35 and 35/36 and the substrates (anterior) were allowed to regenerate for twenty-three days. At that time the anterior eight segments were removed and discarded. Of the surviving substrates (8/9–34/35 or 35/36 + a tail regenerate), four regenerated heads anteriorly. Three were hypomeric with six segments each, and one was hypermeric with nine segments (+ 1).

In series E58 the last ten segments were removed from specimens having one hundred or more segments. At the end of twenty-two days' regeneration, anterior portions were removed so as to leave ten or fifteen segments of the original substrates along with the tail regenerates. One of these small substrates had already produced a bud at the anterior cut surface by the seventh day, at which time circumstances compelled termination of the experiment. Anterior regeneration in

this series, would, if completed, have taken place at levels behind 75/76, while normal posterior substrates, unconditioned by a previous regeneration, failed to regenerate at levels behind 54/55.

In series E49 the posterior portion of the body was removed at 70/71. The anterior substrates were allowed to regenerate posteriorly for eighty days. At that time the tail regenerates were removed at the level of regeneration. One such tail regenerate, then acting as substrate, produced in twenty-seven days, at the anterior cut surface (level 70/71 with reference to location on axis of original worm), a heteromorphic tail, unpigmented but with six setigerous segments and a small anal region without indication of production of further segments. Final substrates in this series were 7 to 10 mm. long and of 30–41 setigerous segments.

C. *After starvation*

To test for the effect of starvation, the following experiment was run (see also series E49 above for regeneration after 80 days' starvation). From worms that had been starved for seventy days or longer, the anterior five or six segments were removed. Each surviving substrate regenerated a hypomeric head (-1 to -3) with metameric differentiation complete and normal.

Discussion

A first step towards obtaining a complete characterization of regenerative capacity in *E. foetida* is determination of the morphological nature of the regenerate produced anteriorly, at each intersegmental level along the axis, by posterior substrates, as well as the number of segments in such regenerates. The latter, often neglected in the past apparently as of little importance, is of some interest with regard to morphogenesis in the Lumbricidae.

Hypermery in head regenerates has now been recorded for the first time in *E. foetida,* and at two different levels, one of which, 8/9, is fairly well back. Hescheler (1896, p. 93) once secured a regenerate with more segments than had been removed but in a series of successive regenerations by a single individual, the worm even then still hypomeric by two segments (removal of 6½ segments, regeneration of 5½; removal of 4, regeneration of 2; removal of 2, regeneration of 3). One hitherto unnoticed case of hypermery in the Lumbricidae has been found—regeneration of four segments after removal of three by a specimen from which the nerve cord had been removed from the next two metameres behind the level of amputation (species unidentified, Goldfarb, 1909, p. 703, Table 4, No. 1.41).

Hypermeric regenerates are of especial interest in connection with the problem of the origin of posterior homoeosis. In *E. foetida* posterior homoeosis of one segment only has been recorded and now in regenerates hypermery of one segment only. As all cases of symmetrical homoeosis in the species can now be considered to have resulted from hypomeric or hypermeric regeneration, postulation of some unknown embryonic cause is no longer necessary.

The new data as to segment number in homomorphic anterior regenerates provides confirmation of the cephalic nature of Michel's and Hescheler's regenerates of seven segments and of Morgan's regenerate of "7 or 8" segments, all of which seem to have been overlooked hitherto.

Presence in a head regenerate of nine segments may indicate a possibility of equimeric regeneration back to 9/10 but is of especial interest in connection with the problem of the constitution of the "head." In the Oligochaeta homomorphic anterior regeneration is generally thought to be restricted to replacement of the "head." The latter, in the Lumbricidae, has been thought to comprise five segments only. Six, seven, and eight (?)-segment head regenerates obtained by Michel and Morgan in *E. foetida* (Table I), and a six-segment regenerate at 9/10, as well as a seven-segment regenerate at 12/13 in *Allolobophora terrestris* (Hescheler, 1896), should have been taken into consideration in this connection. Carpenter's (1948) regenerates of six segments (Table I), and the author's of six to nine, show that regeneration of heads with more than five segments is not exceptionally rare. Smaller numbers in previous work may have been due to less favorable conditions.[1]

The maximum number of segments now recorded for head regenerates in the family Lumbricidae is nine. With the exception of one pair of seminal vesicles and of spermathecae, both of which develop in connection with septum 9/10, reproductive organs in the Lumbricidae are in the region from x posteriorly. All of the evidence available still indicates that Lumbricids regenerate anteriorly only a pregonadal portion of the body. Regeneration, after amputation of the gonadal region, accordingly, is not sufficiently "complete" to enable an individual to reproduce. Although this has often been thought to be characteristic of earthworms generally, at present it appears to be applicable only to the Lumbricidae. In those representatives of other families that have been studied, regeneration of the gonadal region not only is possible but even usual (see Janda, 1926, for the Glossoscolecid *Criodrilus lacuum,* and Gates, 1941, for the Megascolecid *Perionyx excavatus*). The pattern of regenerative capacity, even with regard to this one matter, accordingly, is not uniform throughout the earthworms.

Such data as are now available with regard to segment number, and in particular "7 or 8" segments in a head regenerate at EL 30/31 (Table I), do not appear to support current ideas as to decline in number of head segments regenerated as level of amputation recedes posteriorly (Hyman, 1940, p. 519) and gradient of head regeneration (Liebmann, 1943, p. 601, Fig. 12).

New data given above as to the morphological nature of anterior regenerates agree with some hitherto overlooked in showing a region of definite bipotential regenerative capacity. On amputation within that region a worm may regenerate either a head or a tail. The individual variation in response to the same stimulus suggests a possibility of experimental modification of the nature of regenerates.

The region of bipotential capacity, according to the author's results, is small and bounded by 20/21 and 23/24. Previous work indicates the possibility of considerable extension of those boundaries. Involved in estimation of the posterior limit of cephalic regeneration are two regenerates at EL 30/31 and EL 50/51 (Morgan, 1899 and 1901). Both, it is important to note, were obtained after rediscovery of heteromorphosis. The cephalic nature of the first was proved from sections. The second, having five metamerically normal segments, presumably was large enough to be easily and correctly identified. Level of the first amputation could have been from 31/32 to 38/39 (see note d, Table I), but was probably in re-

[1] Carpenter now reports obtaining in one series, seven six-segment, two seven-segment, and one eight-segment head regenerates at 10/11.

gion of 32/33–35/36. The other amputation, estimated to be at the middle of the body, could have been from 41/42–64/65 (see note d, Table I), but with probability of location at or even in front of 41/42. A level about midway between 30/31 and 40/41, i.e., 35/36, appears at present to be as good an estimate as is possible in the circumstances.

The anterior boundary for heteromorphic tails is extended to 18/19 by Dimon's results (Table I). However, some of her "uncertain" regenerates at 17/18–15/16 presumably had, in absence of all reference to monstrosity, similarities to caudal regenerates. Morgan also had a regenerate at 15/16 thought to be "possibly a new tail" (1902, p. 579). All this seems to warrant placing the anterior boundary provisionally at 15/16. It is also noteworthy that at several still more anterior levels, to 10/11, some regenerates were "imperfect" or "very imperfect," characterizations apparently applied also to regenerates later found to be caudal.

Gradient of segment number in heteromorphic tail regeneration appears, from the author's data, to be of an inverted V-shape rather than the even slope apparently anticipated by Morgan (1902, p. 577) from results obtained on small fragments.

Failure, in the author's experiments, of normal worms to regenerate at levels behind 54/55 was unexpected in view of the results obtained from substrates as small as L14S in *A. terrestris* (Korschelt, 1898, p. 80). Regeneration by tail regenerates from levels behind 54/55 (E49), and behind that level by substrates conditioned by a previous regeneration (E58), suggests a possibility that failures on normal specimens were due to unfavorable conditions.

Regeneration of heteromorphic tails anterior to 20/21 and of heads behind that level, and more important, of both heads and tails from the same levels, does not appear to be in accordance with Liebmann's hypothesis (1943) that specifically polarized, eleocytic aggregates in the coelomic cavities, a head aggregate in v-xx and tail aggregates behind 20/21, determine the nature of the regenerate.

Summary

Posterior substrates of *E. foetida,* cut exactly at intersegmental furrows, regenerated homomorphic heads at levels 1/2–23/24, with equimery at 1/2–8/9 and hypermery (+ 1) at 4/5 and 8/9. Heteromorphic tails regenerated at 20/21 and from 23/24 to 54/55. Behind 54/55, regeneration of heteromorphic tails was obtained only from tail regenerates and substrates conditioned by a previous regeneration. Gradient of segment number in heteromorphic tails appears to be of an inverted V-shape.

Starvation for 70 + days did not inhibit regeneration at 5/6 and 6/7 but all regenerates were hypomeric.

Hypermery and hypomery provide an adequate explanation of the origin of symmetrical homoeosis.

Regenerative capacity in a region from 20/21 to 23/24 is characterized as bipotential since an anterior regenerate, in that region, may be a homomorphic head or a heteromorphic tail.

Review of previous work on *E. foetida* provides indications that the region of bipotential regenerative capacity is even more extensive, with anterior limit of heteromorphosis possibly at or even in front of 15/16 and posterior limit of homomorphosis in region of 35/36.

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FREE-ENERGY RELATIONS AND CONTRACTION OF ACTOMYOSIN [1]

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There are two approaches to muscle. One is that of the physiologist, who studies function hoping to understand the nature and reactions of minute structural elements. The other is that of the biochemist, who studies minute structural elements hoping to understand function. The physiologist carefully preserves structure and subtle qualities; the biochemist wilfully destroys them. This destruction may go as far as the dissolution of the system into single molecules. This approach was that of the author's laboratory, which has shown that the contractile matter of muscle is built of two proteins, actin (F. B. Straub 1942, 1943) and myosin.

Destruction need not necessarily go that far. It may be limited to partial dissolution, which leaves the contractile matter and its geometry untouched, or it may simply consist in the disturbance of certain equilibria by thermal or chemical means. In these cases, it is still convenient to call the system "muscle," but it should be clearly understood that by using this word no attempt is made to confuse such a partial system with the whole living and intact machinery.

In themselves, neither myosin nor actin is contractile. If brought together in a suitable ionic milieu they unite to a complex: "actomyosin." According to the concentration and the nature of ions present, the actomyosin may be charged by the ATP and dissociate reversibly into its two components, or else it may be discharged and dehydrated excessively. If this reaction takes place in a heterogeneous suspension, the actomyosin is precipitated. Owing to its violence, this precipitation was termed "superprecipitation" to distinguish it from the weaker dehydration and precipitation induced by salts alone in absence of ATP. If this reaction takes place in an actomyosin gel, it will lead to excessive shrinking, syneresis. If the elongated actomyosin particles are oriented, the shrinking will be anisodiametric and the gel shrinks in the direction of the axis of the particles and expands at right angles to this direction. Actomyosin threads or muscle fibres, under these conditions, may become shorter and wider without changing their volume. If the reaction takes place in the muscle fibre where the elongated actomyosin filaments form a continuous system, the shortening will be able to do work by lifting weights, or develop tension under isometric conditions, and is usually called "contraction."

The study of these phenomena suggested (see Szent-Györgyi, 1947) that the

[1] This research has been sponsored by a grant from the American Heart Association.

contractile matter is built of functional units, "autones," and that contraction is an "all-or-none equilibrium reaction" of these autones, dependent on temperature.[2] Contraction, *i.e.* the dimensional change, in all probability, is secondary to another change in which charges are neutralized. The size of "autones" is independent of the colloidal particle size (1–1, 5 × 10^6 g) into which myosin breaks up on extraction, and can be expected to be much smaller than this latter. Supposing that the actual shortening is proportional to the number of reacting units, the relative number of charged and discharged units (that is the equilibrium-constant K) was calculated from the macroscopic length of the system.

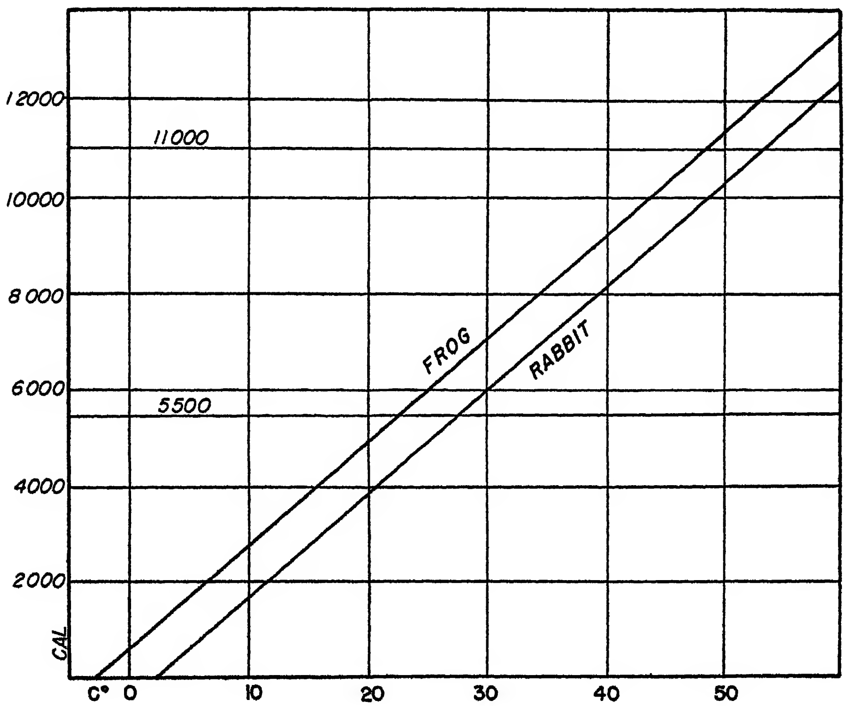

FIGURE 1. The △ F curve of the contraction of extracted frog and rabbit muscle and of frog and rabbit actomyosin threads. Ordinate: △ F in calories. Abscissa: temperature in centigrades (quoted from L. Varga).

From the temperature-dependence of K, the △ F, i.e. the difference of the free-energy content of contracted and relaxed autones, was calculated. The final results of these calculations were summed up by L. Varga (1946) in a curve reproduced in Figure 1. The curve shows that the free energy of the system drops in contraction and that the extent of this drop depends on temperature. In the rabbit it reaches 11,000 calories at 53° C., is 7–8000 calories at 37°, and is 0 at about 0° C.

[2] Buchtal and Knappeis pointed out in 1943 that certain mechanical features of muscular contraction are in accordance with the assumption that the fibre is built of smaller units contracting in an "all-or-none" fashion.

In the frog the same values were reached at 5° C. lower. The curve shows that the relaxed state is the high-energy metastable state, the contracted state the low-energy stable state, contraction being a spontaneous process.

In the first part of this paper material and methods will be discussed. In the second part, the theory will be tested along different lines, and in the third part, the observations will be extended.

Part I: Material and Methods

Muscle is a very heterogeneous tissue. Not only are there different kinds of muscle (smooth, heart and cross-striated muscle), but there are considerable differences between the different muscles of the same sort within the same animal.

There is considerable difference in geometry between the various body muscles. In one muscle the fibres are parallel, while in others they follow a more complicated course, making evaluation of energy relations difficult.

There is considerable difference, also, in the composition of various muscles. The contractile matter, actomyosin, is in its relaxed condition a soft gel which could easily be damaged by mechanical injury were it not protected by connective material, fasciae, collagen fibres and a sarcolem. Muscles lying closer to the surface will need more protection, and in these we will find strongly developed connective material and sarcolem.[3] An almost ideal material for the study of the contractile matter is the musculus psoas of the rabbit. This muscle lies sheltered in the body cavity, protected on one side by the vertebral column, and by the viscera of the belly on the other. Consequently, it contains very little connective tissue, and the sarcolem is poorly developed, which makes the elastic properties of the contractile matter come to the fore. It is built of very long, parallel fibres, stretching from one end of the muscle to the other—fibres which, owing to the poverty of connective material, can easily be separated. It is easy to secure from a medium-sized rabbit very thin fibre bundles 8–10 cm. long which, if necessary, can be decomposed into single fibres of this length. Though occasionally frog sartorius and rabbit m. gracilis were also used, the major part of the experiments reported here were performed on the psoas.

According to the theory outlined, contraction is a spontaneous process going hand-in-hand with a drop of free energy. Thus, contraction should occur spontaneously wherever the ATP-actomyosin system is present in a suitable ionic milieu, and the system should persist in the low-energy stable contracted state. This is actually what happens any time we add ATP to an actomyosin gel or to muscle extracted with water. In the intact resting muscle, however, we find ATP in an active form, linked to actomyosin (see below), but still the system does not contract—contraction being inhibited by some unknown mechanism. If we want the muscle to go over into the contracted state, we have to abolish this inhibition. In the intact muscle this can be achieved by an electric shock or a "wave of excitation." These actions are fleeting and depend on subtle qualities of muscle, on "excitability," which makes them unfit for our present purpose. In order to study equilibria of energy relations, the inhibition had to be removed permanently and uniformly

[3] Ramsey and Street (1940), working with single fibres of the musculus semimembranosus of the frog, found the elastic properties of the contractile matter in resting muscle entirely covered up by the elastic properties of the sarcolem.

throughout the whole mass of the muscle, and the whole contractile matter made to go over into and remain in the contracted state. Poisons like caffeine, quinine, monojodo-acetic acid or chloroform, known to produce contracture, were found unsatisfactory because the tensions developed are very small, showing that only a small fraction of the contractile substance is at any time in the contracted state.

A satisfactory method of abolishing inhibitions is freezing with subsequent thawing, which method also has the advantage that the muscle can be kept in the frozen state, packed in dry ice, for days with undiminished contractility.

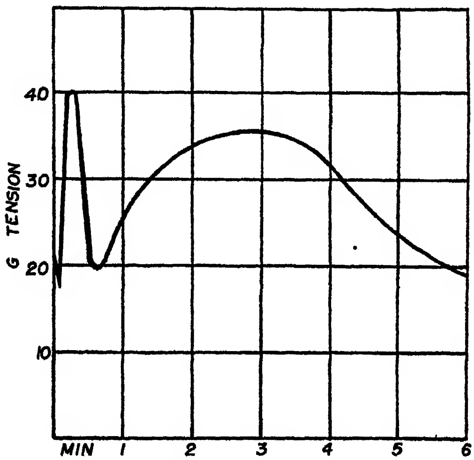

FIGURE 2. Isometric contraction of the frozen sartorius of the frog on thawing. The frozen muscle was immersed into Ringer of 20° C. at 0 min.

The experimental procedure was the following: The rabbit was killed by decapitation, quickly skinned, eviscerated, and the front and the sidewalls of the belly cut off. This exposed the psoas which was liberated from its surroundings. The muscle was decomposed into smaller bundles by punching it through with a small forceps with closed tips and moving the forceps up and down while the index finger of the other hand kept the muscle somewhat lifted. If necessary, ligatures were put on the two ends of the muscle strip. Owing to the poverty of connective tissue, the muscle is rather soft and is easily cut through by ligatures. For this reason, relatively soft and thick threads were applied in dry condition (pearl cotton No. 5).

If frozen strips were desired, fibre bundles of 2–3 mm. diameter were secured, placed on a celluloid ruler (to which the muscle does not stick), stretched to their rest length, the ligature being fixed by artery clamps. Then the strips were covered with freshly powdered dry ice. The strips used were mostly of the thickness of an average frog sartorius, weighing about 40 mg. per cm. Since at higher temperatures the muscle, after freezing, is rapidly damaged, it is important that it should reach temperature equilibrium quickly. So if experiments had to be performed above 30° C., even thinner strips were used weighing 25 mg. per cm.

On thawing, the frozen muscle, if containing the physiological amounts of ATP, contracts rapidly and develops maximal tension.

There are two phenomena which tend to disturb measurements. If the frozen muscle is suddenly placed into Ringer of room temperature, one side may thaw faster than the other and contract suddenly, which causes strong bending which damages the fibres. For this reason, the muscle was allowed to thaw first in 0° Ringer before being transferred into a warmer Ringer of more than 15° C. The first movement of the lever indicates complete thawing which may take place in ten to sixty seconds.

The contraction, elicited by the freezing and subsequent thawing, is developed relatively slowly while the sudden change in temperature may act, in itself, as an impulse and elicit a fast contraction. In this way, in frog muscle, a double peak is obtained, the second of which is mostly lower than the first (Fig. 2). In rabbit psoas this double contraction is less pronounced but still present. Below 10° C. "excitability" is low and the two waves fuse. At higher temperatures they can be made to fuse by making the temperature change less sudden by allowing the muscle to thaw in Ringer of 0° C. before applying the higher temperature Tension given to the muscle also promotes fusion of the two peaks. The same is favored, also, by a thinner diameter Correct values of maximal work or tension can be obtained only if the two contractions fuse into one.

In order to show whether an observed effect was actually due to an interaction of ATP and actomyosin, the latter had to be prepared free of ATP. The effects observed on addition of ATP could then safely be ascribed to an interaction of the protein and the nucleotide.[4]

Thus muscle fibres had to be prepared, free of ATP, and made permeable to this substance, without destroying the actomyosin structure. In earlier work this was done by extracting the muscle with water. In pure water, however, even at low temperature, muscle fibres preserve their full contractility only for a short time.

Satisfactory results were obtained by employing a 50 per cent solution of glycerol. The fibre bundles, once extracted, can be preserved for weeks in this solvent at − 20° C. with undiminished contractility. The psoas was decomposed *in situ* into fibre bundles of about one millimeter in diameter. A thin stick was laid alongside, and four or five such bundles were tied to it at both ends and cut out. In this way straight fibre bundles of rest length and attached to the stick were secured. If bundles of equilibrium length were desired, only one end of the bundle was fixed to the stick and the other end cut, whereupon the muscle contracted to its equilibrium length. Then the other end of the bundle was fixed to the stick. In order to measure the difference between rest length and equilibrium length, a ligature was put on the free end of the bundle before cutting it, and the distance between the two ligatures was measured before and after cutting.

The bundle, tied to the stick, was placed into 50 per cent glycerol of 0° for twenty-four hours. Then the two ends of the muscle with the ligatures were cut off, whereby the muscle, detached from the stick, fell into the single bundles. The muscle was left in this condition for another day at 0° in 50 per cent glycerol and then transferred in this solvent into the deep freezer kept at − 20° C.

[4] Threads prepared from actomyosin are unfit as material for any experiment in which tensile strength is involved, since on extraction the continuous actomyosin filaments present in muscle are broken up, and actomyosin threads contain only their fragments. As will be shown later, one of the actions of ATP is to enable the actomyosin particles to slip alongside one another. Therefore, if an actomyosin thread is loaded or subjected to tension, and ATP is added, the actomyosin particles will contract, as they do in muscle, but they will also slip, and in spite of the contraction (observable in unloaded threads), the system will lengthen. This lengthening has led Buchtal, Deutsch, Knappeis and Petersen (1947) as well as Astbury, Perry and Reed (1948) to the erroneous conclusion that phenomena in actomyosin threads are fundamentally different from those in muscle.

The muscle in 50 per cent glycerol is too stiff to be decomposed into smaller bundles without straining, which causes the bundles to curl up. In water the muscle is too soft. For this reason, before the experiment, the bundles were transferred from 50 per cent glycerol to 20 per cent glycerol for an hour or so and decomposed here to the desired diameter, mostly into strips of 0.2 – 0.5 mm. diameter.

The psoas is built of smaller fascicles, and it is well to follow the outlines of these preformed bundles in decomposing the muscle. The dissecting was done by means of a pair of fine tweezers, used by watchmakers. The ends of the bundles are caught with these tweezers. By pulling them apart, the bundles can readily be separated. Before being subjected to experiment, the fibre bundles were examined under the microscope for continuity.

The experiments were performed in Ringer containing 0.001 M $MgCl_2$. In all experiments glass-distilled water was used because of the deleterious action of copper usually present in common distilled water.

Glycerol-treated fibre bundles of rest length and of 0.1 – 0.2 mm. diameter, if placed into a 0.25 per cent ATP solution, contract rapidly. Diffusion being the limiting factor, the rate of contraction depends also on the diameter. Unloaded fibres contract at room temperature to one-fourth or one-fifth of their rest length. If connected to the isometric lever, on addition of ATP they develop tension comparable in intensity to that developed by intact muscle on maximal excitation. If loaded they will also lift weights isotonically, similarly to intact muscle fibre bundles of similar dimensions.

This contraction of glycerol-treated muscle fibres under influence of ATP is one of the most striking biological phenomena and is very suitable for classroom experiments. Instead of ATP a freshly prepared boiled muscle juice may be used, or an ATP solution may be used prepared by elution of dried, alcohol-precipitated muscle. A smallish rabbit will provide material for a big class. Most of the experiments reported here were done by simple means and are suited for classroom experiments. Some of them have been repeated by the physiology class at the Marine Biological Laboratory at Woods Hole.

In several experiments the maximal total amount of work had to be measured. Theoretically, this can be done in the following way: The muscle is connected to the isometric lever, made to contract, and the tension is measured. Then the muscle is allowed to shorten slightly and made to contract, and the tension measured, etc., till the muscle has contracted maximally and develops no more tension. If the length is plotted against tension, the area between length and tension represents the total amount of work. Such an experiment was performed on the frozen sartorius after thawing. Its result is schematically reproduced in Figure 3, where the hatched zone is the total amount of work. This experiment is a rather difficult one and can be performed only with limited materal and under specific conditions. In the rabbit psoas, contractility is lost after thawing even at 0° C. in fifteen minutes, which makes the experiment impossible. In the frog the experiment can be done at low temperatures only, contractility being lost rapidly at higher temperatures.

A simpler method had to be found which could be applied in any material in a wider range of temperatures. Two such methods are suggested by Figure 3. In this figure the total amount of work is equal to the area BDF, which is one-half of the area BDFH, and the double of the area CDEG. Accordingly, we could measure the total amount of work in two different ways: (*1*) by measuring the maximum tension developed (DF) and multiplying it by the amount of maximum shortening

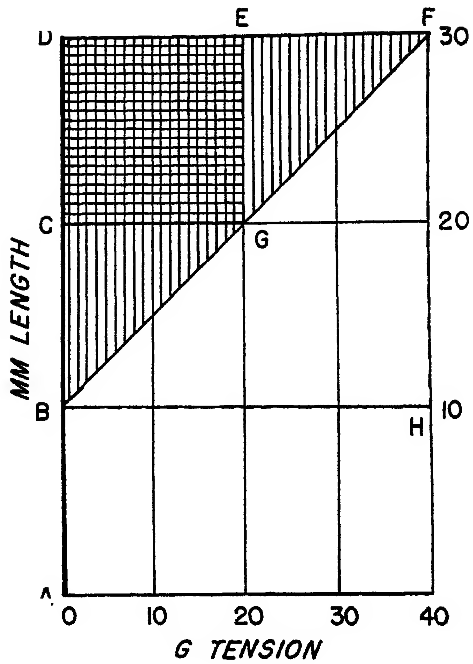

FIGURE 3 Schematic representation of muscular contraction (Sartorius of the frog. Temp. 25° C) AD = initial rest-length, DF = maximum tension, AB = length of maximally contracted muscle, unloaded. Area BDF = total amount of work. BF = length-tension diagram of the excited muscle.

of the unloaded muscle (BD), then dividing the result by two. Psoas strips, after freezing and thawing, contract at room temperature by two-thirds of their rest length. The formula will thus be $\frac{2/3\ \mathrm{lt}}{2} = 1/3\ \mathrm{lt}$ (l = rest length, t = maximum tension). A. V. Hill (1913) developed a similar formula which in his case (frog muscle excited electrically at 0°) was 1/6 lt. (*2*) Load the muscle with the weight corresponding to one-half of the maximum tension (DE), measure the distance by which the weight is lifted (EG), and take the double of the product of these two magnitudes (the area CDEG). The product will be the biggest if the weight is just one-half of the maximum tension, but a small deviation from this value will not cause a considerable error making the area bigger in one dimension and smaller in the other.

The first will be called the "isometric," the second, the "isotonic" method. Both methods may be criticized as to their exactness. The object of the present research is not to obtain exact numeric values, but to obtain information about the basic truth of the theory outlined.

Part II

Observation on heat contracture

According to Figure 1, the Δ F, i.e., the free energy spent by the single units, rises with increasing temperature. The free energy of the phosphate bond in ATP

is 11,000 calories (Meyerhof, 1944) and according to the theory discussed, this energy is needed for relaxation. According to the curve in Figure 1, the expenditure of energy in contraction reaches 11,000 calories at 47° in the frog and at 53° in the rabbit, and exceeds 11,000 calories above these temperatures. If the theory is correct, therefore, the muscle should be unable to relax at these temperatures and should persist in the fully contracted state.

In the frog, experiments were performed in the following way: the sartorius of *Rana pipiens* was provided with a ligature at both ends, was excised and loaded in one series with 2 g. (Fig. 4, circles) and in another series with 20 g. (triangles). The length between ligatures was measured and the muscle dipped into Ringer solution of varying temperature. Above 40° a rapid contraction ensued which was measured at the end of the second minute. The contraction reached its maximum at 47° C. The muscle remained in this maximally contracted state. The gradual lengthening at higher temperatures is due to the denaturation which takes place above 47° C. rather rapidly.

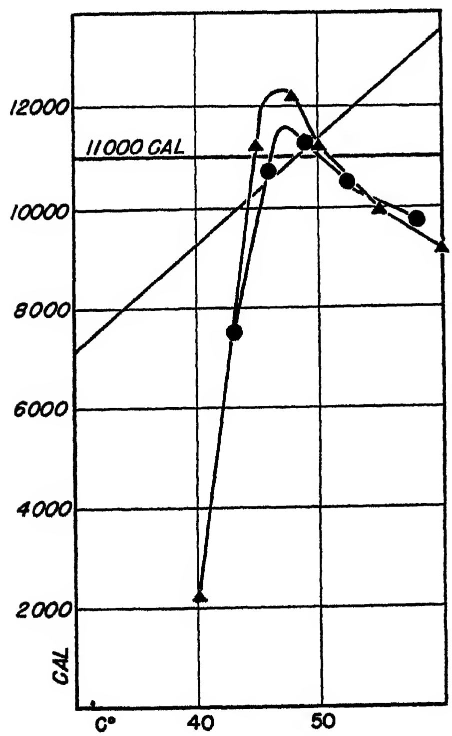

FIGURE 4.

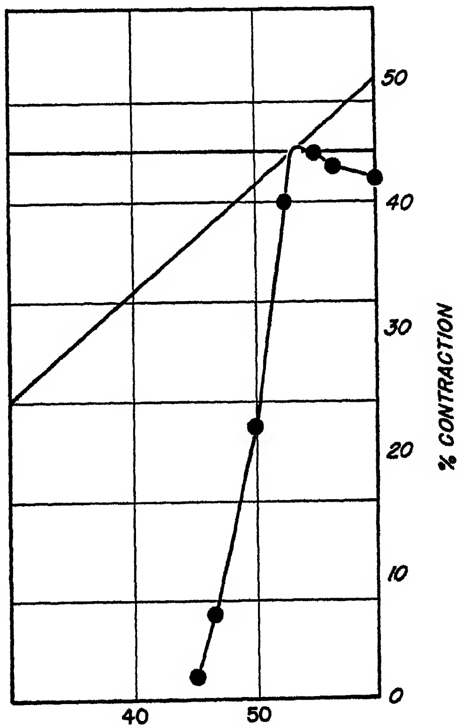

FIGURE 5.

FIGURE 4. Heat contracture in the frog sartorius. The coordinate net corresponds to the right-hand side of Figure 1. The sloping straight line is the Δ F curve, and corresponds to the left-hand side ordinate. The points mark per cent of shortening and relate to the right-hand side ordinate of Figure 4. Abscissa: temperature in centrigrades.

FIGURE 5. Same as Figure 2. Strips of the musculus gracilis of the rabbit.

In the rabbit, experiments were performed in a similar way with the smaller weight (Fig. 5). Experiments were performed with strips of the musculus gracilis similar in dimensions to the frog sartorius. These strips were cut parallel to the fibres. The experiment was performed soon after the animal's death. The results were similar to those obtained in the frog. In both cases the muscle went into permanent maximal contraction at the temperature where the Δ F curve cuts the 11,000 calorie level, as demanded by the theory.

The fact that maximum and permanent contracture was reached only where the expenditure of F was 11,000 shows that the transference of energy from ATP to the contractile system goes without considerable loss.

This heat contracture must not be confused with the shortening of muscle due to heat denaturation. If a muscle is immersed into Ringer of 70° C. an extensive shortening is produced which is not due to the mobilization of the normal mechanism of contraction, but to denaturation. The difference between the two processes can easily be demonstrated. If the muscle is stored a few hours after death at room temperature or overnight in the ice box, the ATP disappears. No rapid contraction will be obtained at 53° in this muscle, but shortening will still be obtained at higher temperatures at which rapid denaturation is produced. This denaturation manifests itself, also, by a turbid appearance. The basic difference between the contraction obtained in the presence of ATP at 53° in the rabbit or 47° in the frog, due to the mobilization of the normal mechanism of contraction, and the shortening produced at higher temperatures and due to denaturation, can be demonstrated, also, by connecting the muscle to

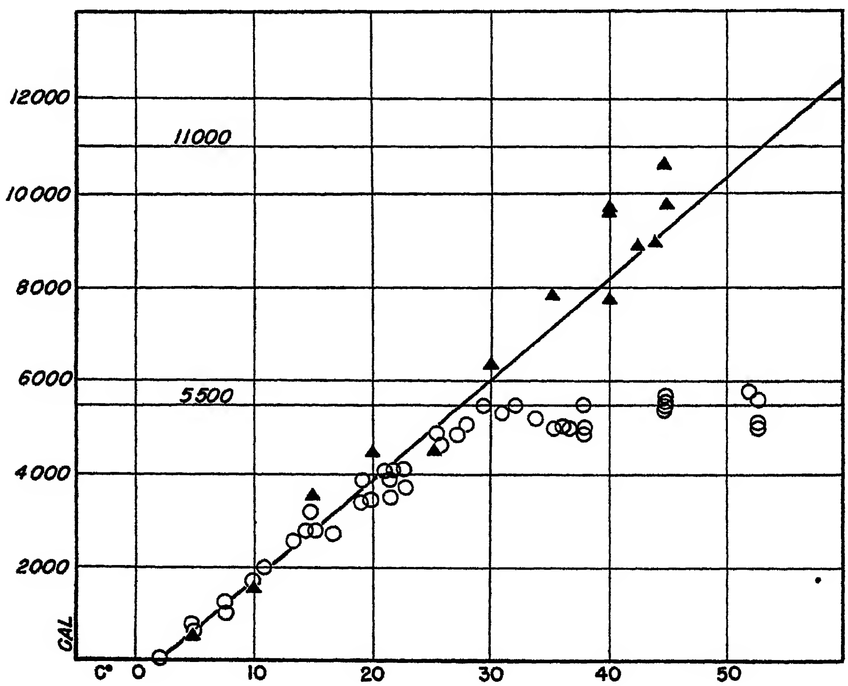

FIGURE 6. The work performed by strips of rabbit psoas, after freezing and thawing, at varied temperature, calculated for 35,000 gm. myosin present. The coordinates are identical with those of Figure 1. The sloping straight line is the theoretical Δ F curve. Triangles: isotonic measurement. Circles: isometric measurements.

the isometric lever. While the theoretical maximal tension is produced in the first case, scarcely any tension is developed in the latter. The gradual lengthening of the loaded muscle above 53 resp. 47° C. is evidently due to the denaturation. This denaturation in the rabbit sets in very rapidly above 53°.

Total work in the psoas

Free energy being, by definition, that amount of energy which can do work, the most direct way of testing our △ F curve is the measurement of the total work at different temperatures.

The amount of work was measured by both the isotonic and the isometric method and the results are reproduced in Figure 6. The psoas strips were loaded or connected to the isometric lever in the frozen condition, a moderate tension being given to the lever. The work done was expressed in calories and calculated for 35,000 g. of myosin. In these calculations the average myosin content of muscle (8 per cent) was taken into account, though it is probable that owing to poverty in connective matter, the psoas contains somewhat more myosin. In order to find out the quantity of myosin present, the muscle was weighed immediately after the measurement was finished, its ends with the ligatures having been cut off.[5]

The results of the isotonic experiments are marked in the figure with triangles. No measurements could be taken above 45° C. owing to the great sensitivity of the muscle to high temperatures after freezing and thawing. As will be seen, the agreement with the △ F curve is satisfactory and pleads for the basic truth of the theory.

The values obtained in the isometric measurement are marked with circles. As the figure shows, at lower temperatures the agreement of the experimental values with the △ F curve is satisfactory. This is true, however, only up to the 5500 calorie level, at which it shows a break to become parallel to the abscissa. This means that the maximum of tension is reached at 28° C. and increases no more if the temperature is raised.

This inability of the muscle to produce the expected tension at these higher temperatures is one instance of the so-called Fenn effect, named after its discoverer who found (1923) that the work done by the muscle depended also on the sort of job the muscle had to do.

The isometric method of calculating total work is based on the assumption that the length-tension diagram is a straight line. It is evident that in this region, where the isometric and isotonic curves differ, the l-t diagram cannot be straight and the method cannot be used. If the results calculated by this method are reproduced, this is because they nevertheless show the fact that on raising the temperature the tension developed remains constant.

The actual l-t diagram of the psoas can be found in this region by loading the frozen muscle strips with different weights, bringing them to the temperature in question, and measuring the maximum distance to which the weights are lifted. The results of such an experiment, calculated for the same weight of muscle, are reproduced in Figure 7.

The △ F curve of frog muscle

Varga's △ F curve admits but a very small expenditure of energy for the frog muscle at 0° C. (600 cal. of the 35,000 gm. unit), which means that at this tem-

[5] After freezing and thawing, contracted muscle rapidly loses weight by pressing out water. This loss may exceed 30 per cent, and is in agreement with the assumption that contraction is connected with loss of charge and hydration.

perature this muscle should be capable only of very feeble motion. It means, also, that the efficiency of the muscle would be exceedingly low because every autone would have to split one high-energy phosphate and pay 11,000 calories for the 600 calories spent.

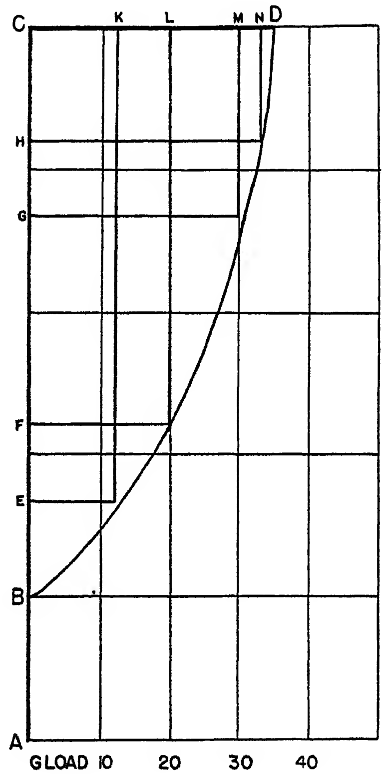

FIGURE 7. Length-tension diagram of strips of the psoas excited by freezing and subsequent thawing at 40° C. AC = length of the muscle; CH, CG, CF and CE are shortening with the weight CN, CM, CL and CK. The curved line connecting the corners of the squares (representing work done) is the l-t diagram.

As pointed out by A. V. Hill in the discussion following the author's lecture at the International Physiological Congress at Oxford (1947), frog muscle at 0° C. is capable of rather strong motion if excited strongly by direct stimulation. Hill had shown previously (1913) that not only is the tension developed by frog muscle at 0° C. rather high, but also the efficiency, which reaches 40 per cent (1939). The author was able to convince himself of the correctness of A. V. Hill's statements. Results of a few experiments on this line are reproduced in Figure 8. They show that the muscle actually spends much greater amounts of energy than allowed by the Δ F curve. The average expenditure around 0° was found to be 4500 calories, which corresponds to a 40 per cent efficiency if the 4500 calories are paid for by

the 11,000.[6] This agreement with Hill's results shows that freezing and thawing yields results similar to electric excitation.

The reason for the discrepancy between Hill's and Varga's results obviously was to be sought in the different nature of the material. Hill worked with intact muscle, Varga with muscle extracted with distilled water and frozen, or with actomyosin threads. It is easy to believe that in the animal the behavior of the actomyosin system is adapted to life at low temperature by some sort of regulation no longer present in extracted muscle or actomyosin.[7] This assumption would become acceptable if it could be shown that the Δ F curve of the whole muscle can be tranformed into the type of Varga's curve by substances which are known to abolish physiological regulations.

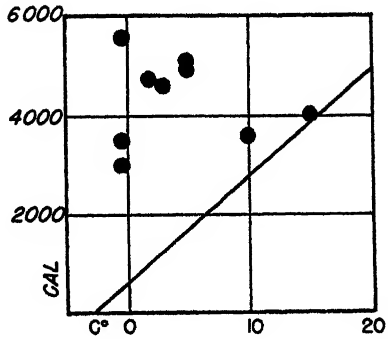

FIGURE 8. Work performed by the frozen sartorius after thawing, calculated for 35,000 gm. of myosin present. Coordinates correspond to lower left corner of Figure 1. Sloping straight line: Δ F curve.

Narcotics, at high concentration, inactivate many physiological mechanisms. Most of them also damage the contractile matter. Chlorated paraffins, however, like ethyl-chloride or chloroform, have no harmful action on actomyosin.

The sartorii of the frog (*Rana pipiens*) were exposed, and provided at their ends with ligatures; the distance between ligatures was noted. The muscle was excised, fixed at its original length and placed for five minutes in Ringer of 0° C., saturated with chloroform. Then the muscle was covered with freshly powdered dry ice and frozen. Its working capacity was measured in the isotonic and isometric experiment at varied temperatures. Also, the Ringer in which the muscle was made to thaw and contract was saturated with chloroform. If the work was measured above 15° C., the muscle was connected to the lever or the weight and allowed to thaw first in Ringer of 0° C., saturated with chloroform, and then transferred to the Ringer of higher temperature.

[6] When the work done by the right and left psoas was compared at slightly varied low temperatures (e.g. 0° and 3° C.), the muscle was found to have a greater Δ F at higher temperature; the slope of the resulting Δ F curve cut the abscissa at about − 30° C., and the 11,000 cal. level slightly under 47°. This suggests that the Δ F curve of intact frog muscle is similar to the curve of actomyosin, but has a different slope.

[7] The experiments were performed between November and January, and thus winter frogs were used.

The work done, and thus the free energy spent, was calculated for 35,000 gm. of myosin. The results are reproduced in Figure 9. The isotonic measurements could not be extended further to the right, above 40° C., the frozen muscle being damaged rather readily by higher temperatures. The action of the chloroform is reversible, and if the chloroform is washed out the work done at lower temperatures increases.

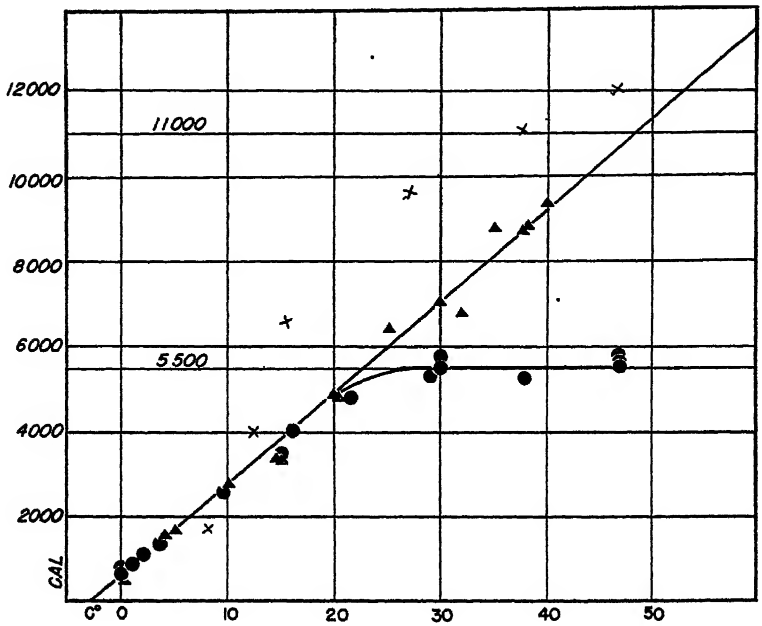

FIGURE 9. Work performed by the sartorius of *Rana pipiens* under influence of chloroform. The curve is analogous to Figure 5. Crosses: isotonic experiment with weights applied prior to freezing.

As the figure shows, the results obtained are similar to those obtained in the rabbit; the curves have the same shape but lie 5° lower. Here, again, the isotonic curve becomes asymptotic on reaching the 5500 calorie level.

In a series of experiments the muscle was loaded prior to freezing with the weight it was to lift later. The results are marked in Figure 9 by crosses connected by a dotted line. No correction was made for the elastic tension, but this correction would be smaller than the actual deviation from Δ F curve, which suggests that the expenditure of energy depends also on the tension which has to be overcome. It was observed repeatedly that the sartorius developed higher tension after freezing and thawing if stretched previously for a short while.

To sum up the experience with frog muscle, we can state that the Δ F curves of whole frog muscle and extracted frog muscle are different, the latter being identical with the Δ F curve of actomyosin threads. By treatment with chloroform, the curve of the whole frog muscle can be transformed reversibly into a curve similar to that obtained by Varga in his extracted material. This brings out the point

that the Δ F curve of actomyosin can be greatly modified by accompanying substances, and opens the possibility of adapting the contractile material to different physiological functions. Actomyosin is not a sharply defined substance and is accompanied by other different substances, proteins and lipins which actually make part of the system, and it is not surprising to find that systems containing different substances may have different Δ F curves. Ionic equilibria, disturbed by extraction, may also contribute to shaping the Δ F curve.

In the glycerol-treated psoas, immersed in 0.2 per cent ATP dissolved in Ringer, the Δ F curve, obtained by the isometric method, cuts the abscissa if extrapolated at $-10°$ C., while if extrapolated towards high temperatures, it cuts the 11,000 caloric level somewhat above 50° and has thus a different slope than untreated muscle.

Thermodynamic reversibility

One of the most important implications of the Δ F curve (Fig. 1) is the thermodynamic reversibility of contraction, which means that the energy spent by the single units in contraction is a function of temperature on which it depends in a reversible way. It should thus be possible to increase or decrease the tension of the contracted muscle solely by variation of the temperature.

Owing to secondary complications, not every material is suitable for this demonstration. The rapid loss of ATP and contractility in the rabbit muscle, after freezing and thawing, rules out this material. In the intact frog muscle, as shown, the effect of temperature is compensated. We can expect to be able to demonstrate

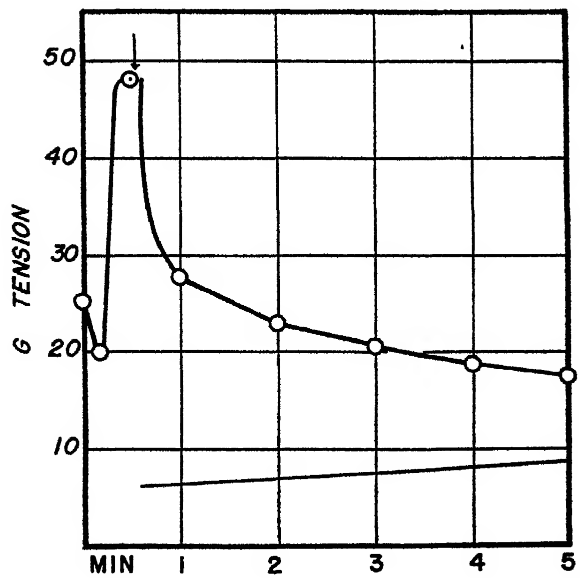

Figure 10. Tension developed by the frog muscle, treated with chloroform, at 13 and 0–1° C. At the arrow the warmer Ringer was substituted by the cold one. Sloping straight line: tension demanded by the Δ F curve.

thermodynamic reversibility in the frog muscle treated with chloroform, or in extracted strips of the psoas immersed in a solution of ATP.

Figure 10 illustrates a result obtained with frog muscle. The sartorius was treated with chloroform and frozen, connected to the isometric lever and dipped into chloroform Ringer at 13° C. After a short negative phase, usually seen in such conditions, the muscle rapidly contracted developing 48 gm. of tension. As the maximum was reached (26 sec.) the Ringer was exchanged for another chloroform Ringer of 0° C. The muscle suddenly relaxed. During the experiment the temperature of the Ringer rose 1° C. The sloping line in Figure 10 shows the theoretical tension of the muscle demanded by the Δ F curve. As can be seen, the tension of the muscle asymptotically approaches this line. The control experiment done with the other sartorius of the same frog showed that if the temperature is kept constant at 13° C., the tension remains high after a slight initial depression and does not fall more than a few per cent in five minutes.

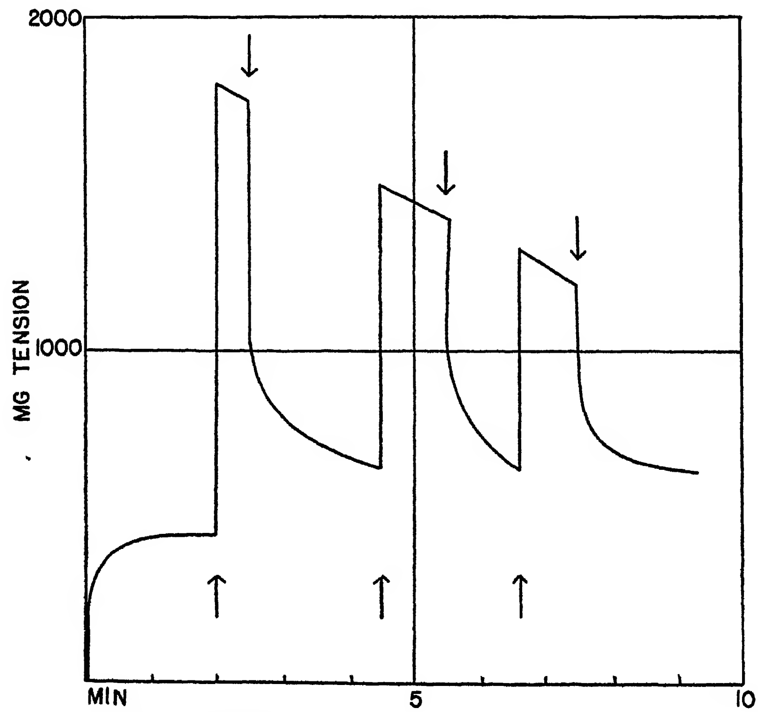

FIGURE 11. Tension developed by extracted psoas fibres in ATP at 25° and 1° C.

The following experiment, reproduced in Figure 11, illustrates thermodynamic reversibility in a fibre bundle of the psoas, treated with glycerol: The fibre bundle (42 mm. long and 0.5 mm. thick) was connected to the isometric lever, a tension of 200 mg. was given, and the muscle immersed in Ringer of 1° C. Then the Ringer was exchanged for another Ringer of the same temperature containing 0.2 per cent ATP. The tension rose to 450 mg. At the arrow pointing upwards, the

fluid was exchanged for an identical solution of 25° C The tension was noted at once and found to be 1800 mg. A reading was taken every thirty seconds and the warm ATP Ringer was exchanged for the cold one (arrow pointing downwards). A reading was taken at once and subsequently every thirty seconds. At the arrow pointing upwards, again, the warmer Ringer was introduced, etc. As can be seen in the figure, the change in tension is immediate and reversible. As the experiment went on, the muscle gradually lost contractility.

The measurement of the diameter has no pretense of accuracy. If the tension is calculated from the final measurement at 25° for one cm.^{2}, a tension of 2½ kg. is obtained, which shows that the tension developed by a glycerol-extracted muscle under the influence of ATP is of the same dimension as the tension developed by an intact muscle under the influence of maximal stimulation.

Part III

Elasticity of the resting psoas

The fresh, resting psoas shows a moderately high elasticity, as demonstrated by the following experiment (Fig. 12): A strip of the psoas of the freshly killed rabbit was connected to the isometric lever, weight 56 mg., rest length 78 mm. (RL in fig.), equilibrium length (EL) immediately after excision, 62 mm. The muscle was slowly stretched, its length being increased by one millimeter in five seconds. After the rest length was reached, the muscle was released for a few seconds and then its equilibrium length measured. This was done by straightening the muscle out, measuring its length, and then applying a tension of 200 mg. and measuring the length again. The difference in length in both measurements was usually 2 mm. In the figure the average of these two measurements is given. After this measurement was completed, the muscle was stretched to the length from which it was released. This stretching was roughly twice as fast as the stretching before. Then the muscle was stretched further at the original lower rate and the procedure repeated after every 5 mm. of additional stretching till the muscle broke. The muscle was kept during the experiment in a wet chamber, immersed in a waterbath of 0° C. In the figure the gradual stretching is symbolized by the upper straight line which refers to the ordinate (mm). The corresponding equilibrium length is reproduced in the middle curve. The single points of this curve lie under the point of the upper curve from which the muscle was released. The lowest curve shows the tension developed on stretching and refers to the ordinate, the numbers of which mean gram-tension in this case. The lowest straight line simply shows a slope of 45° and means that if the curve of tension is parallel to this line, the muscle obeys Hooke's law.

The upper line shows that the muscle broke when extended to 173 per cent of its equilibrium length, and the middle curve shows that this extension was elastic in the whole range of measurements. The middle curve illustrates the well known fact of the poor reproducibility of the equilibrium length. The lowest curve shows that up to the rest length the contracted units can be stretched practically without resistance, but begin to develop resistance at this point. If the muscle were *in vivo* at its equilibrium length, it could develop no tension at the beginning of contraction; if it were tensed any more, it would be spastic.

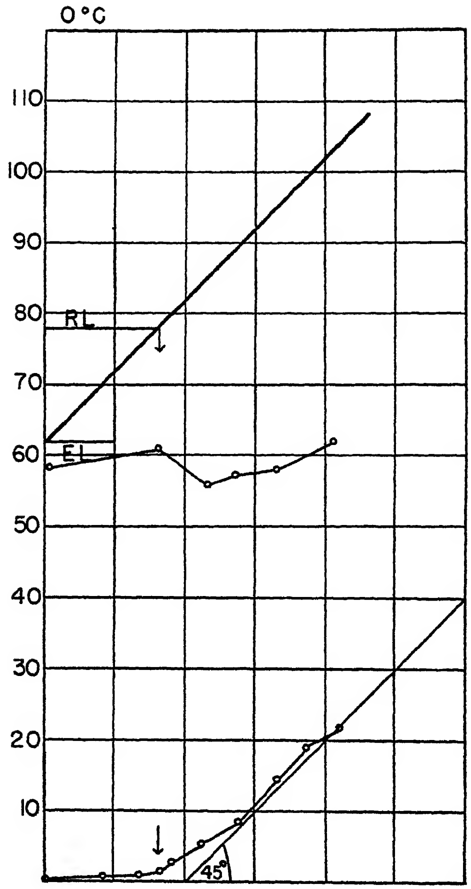

FIGURE 12. Length-tension relations of the psoas at 0° C. (see text).

In Figure 13 an identical experiment is reproduced, performed at 23° C., weight of the muscle 130 mg.

In this experiment, the muscle broke when extension reached 190 per cent of the equilibrium length. As the middle curve shows, this extension is, at its higher degrees, not completely reversible, and the elastic part of the extension is but 163 per cent of the equilibrium length. The middle curve shows the rest length to be better reproducible at this temperature. The lowest curve again shows the contracted units to be extensible at the beginning with practically no resistance. The middle part of the curve obeys Hooke's law; the upper part shows excessive tension. The transition from the region of low tension into the Hooke region is rather sharp and corresponds to the rest length. If the relaxed units contract, the tension developed will be proportional to the contraction from the beginning which makes precise motion possible. On the other hand, having practically no tension, they will not impede the motion of their antagonist.

The muscle obeys Hooke's law up to one-half of the maximum of tension. At the point where it begins to develop excessive tension, stretching begins to be inelastic, causing slipping and permanent damage to the muscle.

Elasticity, ATP, and the slope of the ΔF curve

Freshly isolated strips of the psoas show high elasticity. After the death of the animal, its ATP gradually disappears in a few hours' time, as shown by M. Borbiro in a separate paper (pp. 162–7, this issue). Parallel to this disappearance of ATP, the elasticity of the muscle declines, and if the muscle is excised four hours after death, it will usually be found entirely inelastic. On stretching, the maximum tension is developed at once, and the muscle tears without considerably increasing its length.

The question arises whether the high elasticity of fresh muscle is actually due to the ATP present, and whether the disappearance of this elasticity can actually be attributed to the decomposition of this nucleotide. It can be shown that this is

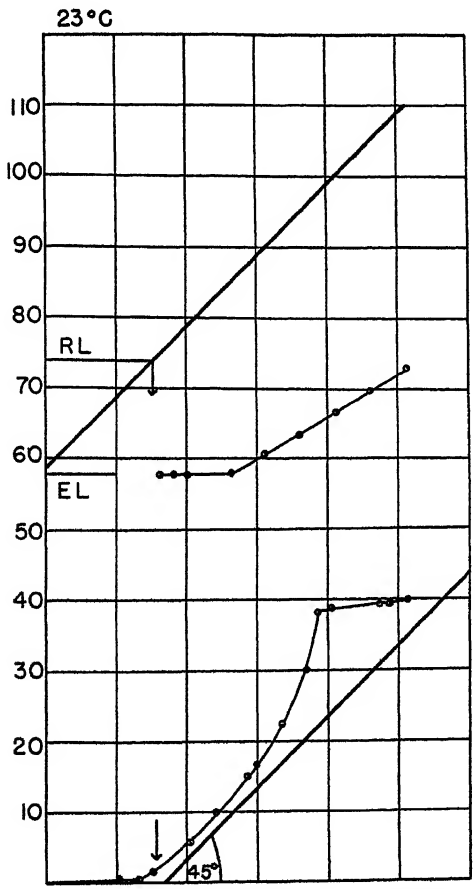

FIGURE 13. Same as Figure 12, at 23° C.

actually the case: if strips of the psoas are extracted at equilibrium length with 50 per cent glycerol, they are found to be entirely inelastic. At 0°, in Ringer, they cannot be stretched at all without breaking, and even at 13° C extensibility does not exceed two per cent. If, however, 0.2 ATP is added to the Ringer, the muscle again becomes extensible. Using fibre bundles of 0.3–0.4 mm. diameter, the muscle could readily be stretched at 0.5–1° C., on an average to 145 per cent of its rest length.[8]

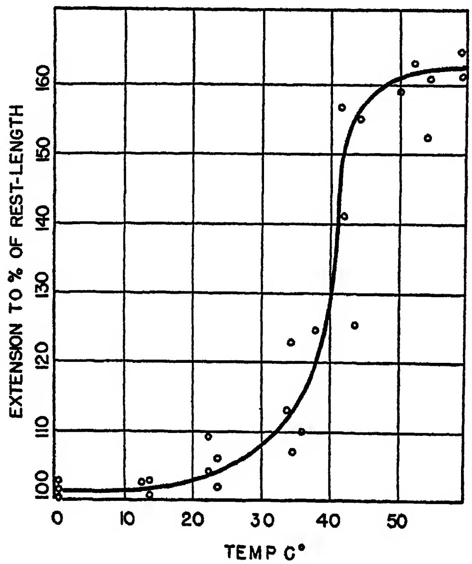

FIGURE 14. Extensibility of extracted psoas fibres at varied temperature 100 per cent of rest length (abscissa) means that the fibres are not extensible

The extensibility of the extracted muscle, in absence of ATP, is a function of temperature (Fig. 14). At 0° the muscle is practically not extensible; extensibility rises slowly with increasing temperature, rising rapidly at body temperature. The extensibility at higher temeperatures, up to 53°, is not due to denaturation, as shown by the relatively big force needed for extension. Denaturation rapidly sets in at a somewhat higher temperature, 54–55°, where the muscle soon becomes plastic, offering practically no resistance to stretching.

This shows that, in absence of ATP, the actomyosin particles in muscle are surrounded by unbalanced forces which link neighboring particles together and make it impossible for them to move relative to one another, turning the system into a rigid, inelastic gel. They are counteracted by heat-agitation.

The extensibility of muscle at low temperatures in presence of ATP shows that the elasticity of muscle actually depends on its ATP, which must be present in the resting state linked to the actomyosin. The interdependence of ATP and elasticity also shows that in the psoas the elastic properties observed were predominantly

[8] These results are in agreement with previous findings of Th. Erdős (1943) on the relation of ATP to rigor mortis.

those of the contractile matter, actomyosin, and were not due to the sarcolem or the connective tissue present, no specific reaction being known to take place between these latter and ATP.

It is evident that free energy is needed to abolish the cohesive forces linking the particles together, and if this is achieved by ATP, so it is also evident that part of the free energy of the ATP-actomyosin system will have to be spent in this reaction. The free energy spent in this reaction will manifest itself in the stability of the link between ATP and the actomyosin. The free energy needed to make actomyosin elastic will decrease with increasing temperature, actomyosin becoming more elastic by itself on elevation of the temperature. This expectation is in agreement with the results of Mommaerts (1941–1942) who found that at low temperatures, the dissociation constant of the actomyosin-ATP complex was exceedingly low. F. B. Straub (1941–42) found that the binding of pyrophosphate to actomyosin greatly depends on temperature, being strongest at the lowest temperature.

We can thus conclude that the free energy of the actomyosin-ATP system is spent in two successive steps. In the first step the ATP is linked to the myosin, cohesive forces are abolished, and a new system is formed in which particles are rendered capable of relative motion, contraction or extension. The free energy spent in this reaction, henceforth called "Reaction I," will decrease with increasing temperature.

The second step, "Reaction II," entails the dimensional change called "contraction." In the resting muscle, we find the actomyosin-ATP system in the state of Reaction I, but Reaction II is inhibited by some unknown mechanism. This inhibition is abolished by "excitation" which causes Reaction II to take place. In the glycerol or water-extracted muscle suspended in ATP this inhibitory mechanism is no longer present, and Reaction I is followed spontaneously by Reaction II.

It is evident that Reaction II can only spend the free energy unspent by Reaction I, which may be involved in the slope of the Δ F curve of contraction (Fig. 1).

Since actomyosin devoid of ATP-ase activity can still contract, as shown by Buchthal, Deutsch, Knappeis and Petersen (1947), we can conclude that no phosphate is liberated in Reaction I or II, and the whole loss of free energy of the system takes place without splitting of high-energy phosphate links.

The increase of extensibility of actomyosin under the influence of ATP was the first known specific effect of ATP on "myosin" discovered by Engelhardt, Ljubimova and Meitina (1941).

If actomyosin is stored in dehydrated condition, links are developed which are not quantitatively split by ATP. Such links develop especially fast in contracted, thus discharged, actomyosin. Their development is favored by parallel setting.

These observations on elasticity and its *post mortem* changes are in agreement with previous findings of Th. Erdös (1943), corroborated and extended by Bate-Smith and Bendall (1947).

The weight of the autones

The Δ F curve (Fig. 1) shows the free-energy change of the single autones at any given temperature. If the weight and myosin content (8 per cent) of the muscle are known and the Δ F curve and measurements of the total work are accepted, the weight of the single autones can be found by simple numeric calculation.

If, for instance, at a given temperature the ΔF curve indicates an expenditure of 5500 calories per unit, and our piece of muscle performed 0.0055 calories' worth of work and contained 35 mg. of myosin, then the weight of the myosin-unit which has spent 5500 calories would have been 35,000 gm., and this would be the unit weight of myosin contained in one autone. As has been shown (Figs. 6 and 9), isotonic measurements and isometric measurements up to the break indicated a unit weight of 35,000 gm. for myosin. Above the break, the isometric experiments do not yield correct values. At the temperature at which the ΔF curve cuts the 11,000 calories level (53° C. in the rabbit and 47° C. in the frog), the unit weight calculated from the work done by the isometric method must be the double of 35,000 gm. In a series of experiments the unit weight of myosin was calculated from the work done by the sartorius as measured by the isometric method at 47° C. The freshly isolated sartorius was in these experiments connected to the isometric lever and dipped into Ringer of 47° C. The results are given in Table 1.

TABLE I

	74,000
	66,000
	74,000
	74,000
	69,000
	70,000
	72,000
Average	72,000

This calculated unit weight of 35,000 gm. is based on the current myosin estimations. Should muscle be found to contain more myosin than 8 per cent, this would mean that the unit weight of myosin taking part in the building of one autone is correspondingly higher. There are indications suggesting that the psoas actually contains more myosin than 8 per cent. Moreover, if there is a loss of free energy, this also entails a bigger unit weight. So 35,000 gm. is rather an order of magnitude and the lower limit than the absolute value, which might be equally well 70,000 gm. H. B. Bull (1946) arrived along different lines at a unit weight of 40,000 gm.

CONSIDERATIONS

It may be asked how far the observations made on the psoas of the rabbit, a specific case, reflect a more general behavior. There are different kinds of muscle with widely different functions and structure. As reported before, the contractile matter of all these different muscles seems to be similar, and actin and myosin prepared from cross-striated, smooth, or heart muscle, or even myomas, can be interchanged to form actomyosin which contracts on addition of ATP. Even clam muscle shows similar reactions (A. Lajta, 1947).

There are indications suggesting that the regularities observed are not limited to the contractile matter. If the muscle is minced soon after death and suspended in an alkaline 0.6 M KCl solution, a sticky extract is obtained which owes its high viscosity to the dissolved fibrous structural protein, actomyosin. The hydration and dissolution of this protein is not merely a result of its interaction with the salt-solution. The ATP present has a decisive influence, and if we store the minced muscle for a few hours prior to extraction, giving time for the decomposition of ATP, the subsequent extraction will yield an extract of low viscosity containing no actomyosin. Addition of ATP will restore conditions found in fresh muscle.

As shown by Lajta (unpublished), kidney and other tissues behave in an analogous way. The fresh mince, if suspended in the alkaline salt solution, yields a sticky, highly viscous extract, and the strong double refraction of flow reveals the presence of dissolved fibrous structural proteins. If, however, the mince is incubated, the subsequent extraction yields a fluid of low viscosity containing no fibrous proteins. During the incubation the labile phosphate present disappears. Contrary to muscle, however, the original condition cannot be restored by the addition of ATP or a fresh boiled juice. The labile phosphate, the disappearance of which seems to be connected with this change, is found to be linked to nucleic acid present in the protein. The nucleic acid, prepared from fresh kidney, shows a high content of labile phosphate.

This behavior is completely analogous to that found in muscle, with the difference that instead of a single nucleotide, ATP, in kidney and other parenchymatous organs we find nucleotides united to long chains, to nucleic acid. In muscle, such long chains would interfere with motility.

The close analogy with muscle suggests that in other organs, too, the protein is built of small functional units, each correlated to a nucleotide which governs its physical state and enables the system to develop the two different states, the high-energy, charged, hydrated state corresponding to rest, and the low-energy level corresponding to activity.

Summary

Material and methods of measurement of physical properties of muscle were discussed.

Heat contracture, total work of muscle, and thermodynamic reversibility were studied and found to be in agreement with earlier assumptions.

Elastic properties of muscle and their relation to ATP were studied.

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ON THE RELATION BETWEEN TENSION AND ATP IN CROSS-STRIATED MUSCLE

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According to the theory outlined in the preceding paper, the contractile matter of muscle is built of functional units containing myosin, actin, and ATP. Since muscle contains no free ATP, it can be expected that if the ATP concentration of muscle decreases, the number of contractile units decreases proportionately. The ATP concentration of muscle decreases after the death of the animal (Th. Erdös, 1943). The object of the present research was to see whether the ATP content and the tension developed by muscle decrease proportionately. Such a parallelism would support the theory outlined, while a lack of parallelism would plead against it. For this reason, we measured, simultaneously, the tension developed by the muscle and the ATP concentration at various intervals after the death of the animal.

The material used was the musculus psoas of the rabbit. At various intervals after the death of the animal, strips of this muscle were cut out and frozen at once. The tension developed on thawing was measured.

The methods hitherto used for the estimation of ATP were found to be unsatisfactory for the following reason: we possess no direct method for the estimation of ATP. When this substance has to be estimated, extracts of the tissue are subjected to limited acid-hydrolysis, and the quantity of ATP is calculated from the quantity of labile phosphate liberated. Muscle contains *in vivo* a not inconsiderable amount of free phosphate. As the ATP is gradually decomposed *post mortem*, the amount of hydrolyzable phosphate decreases while the amount of free phosphate increases, and thus a slight error in the phosphate estimation makes the results of the ATP estimation doubtful. A new method of phosphate estimation had to be constructed in which the free phosphate did not interfere with the estimation of the ATP.

In the first part of this paper this method will be described. In the second part, the results obtained by this method will be given.

Method of Phosphate Estimation

The method is based on the ready solubility of phosphomolybdic acid in iso-butyl alcohol, described by Berenblum and Chain (1938), and on the yellow color with which the acid dissolves in this reagent. The muscle was extracted with trichloracetic acid. Ammonium molybdate was added to the solution. The free phosphate present combined with the molybdate and was shaken out with a mixture

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of iso-butyl alcohol and ethyl ether. Then the fluid was hydrolyzed and the free phosphate shaken out with iso-butyl alcohol and estimated colorimetrically.

After the trichloracetic acid extract of the muscle is shaken out with butyl alcohol ether, it still contains a small quantity of phosphate. This quantity can be estimated and taken into account. If the extract is shaken out a second time with alcohol ether, no phosphate is left, and no correction has to be made on the final readings. In the present paper the former method was used.[8]

Muscle extract contains substances which, after boiling with HCl, yield products which interfere with the development of the yellow color. These substances are eliminated by the alcohol ether, since they are of lipoidic nature.

The detailed description of the procedure is as follows: the rabbit (2–3 kg.) was decapitated, eviscerated, the side walls of the abdomen cut off and the psoas exposed. Two thin strips of the psoas were taken out, provided with ligatures, fixed and frozen with dry ice at their resting length, as described in the preceding paper. These strips were used for estimating the maximum tension developed by the muscle on thawing at 15° C. Simultaneously, a somewhat thicker strip of about one gram weight was cut out from the same region, weighed and frozen. This strip served as material for the ATP estimation. The remainder of the muscle, left *in situ,* was covered with cotton wool wetted with Ringer. The procedure was repeated once every hour. First the right and then the left psoas was used. Such samples were taken until the muscle showed no elasticity and no contractility after thawing. One hour later a last sample was taken.

Extraction: 25 ml. of 10 per cent trichloracetic acid was pipetted into a mortar which was pre-cooled to – 20° C. The fluid solidified to a brei. The muscle, after having been weighed, was placed into the brei in frozen condition and ground to a fine suspension. On thawing, the suspension was transferred into a centrifuge tube and spun. The clear fluid was poured into a 50 ml. graduated measuring cylinder provided with a ground glass stopper. The volume was noted; then for every 10 ml., 1 ml. of 10 per cent ammonium molybdate solution was added and the fluid mixed. Then 1 ml. of iso-butyl alcohol was added for every 4 ml. of the fluid, and 4 ml. of ether added for every ml. of butyl alcohol used. The fluid was strongly shaken for twenty-five seconds and allowed to separate. If there was no ready separation of the two phases, the fluid was centrifuged. Then the ether butyl alcohol mixture was sucked off through a capillary glass tube. A few ml. of ether were added without shaking in order to wash off the remaining alcohol ether. The volume of the fluid was noted. If, after the shaking with alcohol ether, a heavy precipitate was formed, this was separated by centrifugation. The fluid was divided into samples, each of which corresponded to 100 mg. of muscle, and pipetted into test tubes. Out of nine samples four were put aside. To five samples, 1/10 parts of concentrated HCl (approximately 10 N) was added and the tubes placed into the boiling water-bath for seven minutes and then rapidly cooled. To the unboiled samples, the same amount of HCl was added. To all tubes one drop of 0.1 per cent potassium permanganate was added which stained the fluid a rose color. This color persisted for about half a minute. This was done in order to oxidize any reducing agent present which would reduce the phosphomolybdate. Then 10 per cent ammonium

[8] If for any reason the quantity of free phosphate present in the muscle extract had to be known, this could be estimated colorimetrically in the combined alcohol ether extracts.

molybdate was added to the unboiled tubes, and 5 ml. iso-butyl alcohol to all samples. The butyl alcohol used here was shaken out previously with water. (This is necessary in order to prevent the butyl alcohol from taking up water later.) The fluid was shaken strongly for five seconds, the opening of the tube being closed by the thumb covered by a rubber glove. After the two phases separated, the watery phase at the bottom was sucked off by means of a thin glass tube, connected to the vacuum by a thin rubber tube which was pinched tight while the tip of the tube was passing the alcohol. Then the alcohol was poured over into the colorimeter tubes which were marked at their 5 ml. volume. Usually the volume of butyl alcohol is less than 5 ml. It was filled up to 5 ml. with butyl alcohol which was used to rinse the tubes that contained the extract previously. Then to every tube 1 ml. of ethyl alcohol was added and the color estimated in the Klett-Summerson colorimeter with the S 42 blue light filter (400–460 mμ).

As a standard, a solution of KH_2PO_4 was used, containing 0.01 mg. per ml. Samples of 1, 2 and 3 ml. of this fluid were filled up with water to 4 ml., 0.5 ml. cc. HCl and 0.5 ml. of 10 per cent molybdate were added; then the fluid was shaken out with 5 ml. butyl alcohol which was treated as described above.

Experimental Results

Before embarking on the problem proper, a few minor points had to be cleared up. First, is the method of P estimation reliable, and is the distribution of ATP in the psoas homogeneous?

A rabbit was killed and six samples of 1 g. were taken from different parts of the two psoas muscles. In Table I the actual colorimeter readings are reproduced. The six upper columns related to the unhydrolyzed extract are thus the zero values. The corresponding readings of the hydrolyzed samples are reproduced in the lower columns.

As can be seen, the readings are very uniform. The one value in the fourth column, marked with an asterisk, is evidently due to some rough mistake and has to be discounted. The other single values do not differ from the average by more than five per cent. The average of the 0 value was substracted from the average of the hydrolyzed product. From this the ATP was calculated. The standard with

Table I

	40	38	30	29	24	29
	39	35	30	69*	24	28
	39	37	30	29	24	28
	202	194	190	204	198	204
	200	189	200	200	194	202
	204	194	198	206	196	202
	190	196	189	200	200	198
	196	199	187	204	208	194
	195	186	198	214	195	195
Average	198	194	194	205	198	199
ATP	3.55	3.50	3.47	3.70	3.68	3.60 mg. per gm.

0.02 mg. phosphate gave a reading of 80. The quantity of P found was multiplied by 8.4 to give the ATP, which is noted in the last horizontal line. This shows the ATP content of the psoas to be very uniform, 3.6 mg. ATP per gm.

According to the literature, muscle contains 2–2.5 mg. ATP per gm., thus considerably less than psoas. This difference is probably due to the shielded position of the psoas and the consequent poverty of connective material. In order to elucidate this point, samples of different muscles of a freshly killed rabbit were taken and subjected to analysis. Results are reproduced in Table II.

TABLE II

Psoas	3.55 (mg. ATP per gm.)
Deep muscles of the back	3.04
Big adductor muscle	2.56
Musculus gracilis	2.10
Superficial muscle of the back	2.10
Smaller muscles from the gluteal region	1.96

These values show that the more superficial the position and the richer the connective tissue, the lower the ATP content. The muscles of the whole animal would give an average of about 2.5 mg. ATP per gm.

The third question which had to be cleared up was whether the ATP content of the psoas decreases uniformly in all its parts after the death of the animal. Preliminary experiments have shown that the rate of disappearance of ATP *post mortem* depends on the temperature and the oxygen supply. If the muscle is cut into thin strips which are exposed to air, the disappearance becomes much slower. While the ATP in the muscle left *in situ* may disappear within three to four hours, muscle strips exposed to air may contain ATP and thus remain contractile at room temperature even twenty-four hours after the death of the animal. Experiment also showed that in the muscle left *in situ,* the ATP disappeared faster in the deeper-lying dorsal than in the superficial ventral part.

Experiment: The rabbit was killed, the psoas exposed as usual and covered with wet cotton wool. Three hours later, five strips, weighing approximately 1 gm., were cut out and analyzed for ATP. One of the strips was taken from the lateral edge of the muscle, two from the ventral surface, two from the deeper-lying dorsal surface. ATP (mg.) per gm:

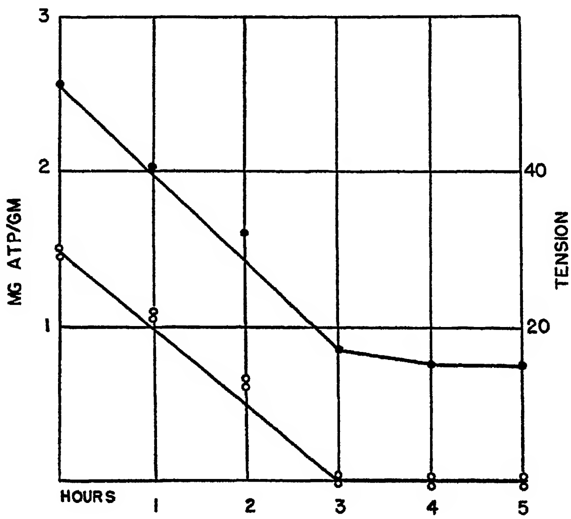
edge:	2.53	
ventral:	2.77	2.77
dorsal:	1.80	1.95

If the ATP content of muscle and its tension are to be measured simultaneously, it is essential that strips from the same region be used for both measurements. Even with this precaution, considerable scattering of results can be expected.

The relation between ATP content and tension was studied in eight experiments. The following example may be cited (Fig. 1): Samples of muscle were taken every hour after the death of the animal. The ATP content in milligrams per gram of muscle is marked in the curve by points. They relate to the left ordinate.

The tension developed is marked with circles and refers to the right-hand side ordinate. (The scale of this ordinate is arbitrary and is chosen in such a way that the numbers, if multiplied by 100, give the total working capacity in calories calculated for 35,000 gm. myosin by the formula: $\frac{1}{3}$ tension $\times$ length $\times$ 0.000023.)

As the curve shows, tension and ATP content run parallel. At the end of the third hour the muscle develops no more tension and does not contract on thawing, and is found to be completely inelastic. At this point, the ATP curve shows a break and becomes roughly parallel to the abscissa.



As the curve shows, the muscle at this point still contains a not inconsiderable amount of labile phosphate. Whether this hydrolyzable ATP is derived from ATP or some other source (ADP?) cannot be stated at present. If this hydrolyzable P is derived from ATP, this ATP must be in some way different from the rest, because it is no longer split by the muscle (or is split only exceedingly slowly) and has no influence on contractility and elasticity. This "residual" hydrolyzable phosphate was found in approximately the same proportion in all experiments.

The second point, equally borne out by the other experiments, is that the decrease of ATP concentration is linear: the rate of its disappearance is independent of its concentration. The most likely interpretation of this rather unexpected fact is that the splitting of ATP depends on some change in the contractile matter. As has been shown by A. Biro and A. E. Szent-Györgyi (unpublished), myosin is enzymatically active in its contracted condition only.

In two out of the eight experiments, the ATP concentration did not fall at all during the first hour after death. This can be explained by the presence of creatinephosphate which rephosphorylates the ADP formed.

All experiments gave similar results. In most of them the scattering was stronger than in the quoted example. Nevertheless, all experiments bore out the close parallelism between tension developed and the quantity of ATP present.

SUMMARY

A new colorimetric method of ATP estimation is described. In the psoas of the rabbit the *post mortem* decomposition of ATP and the loss of contractility are parallel.

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EARLY STAGES IN THE LIFE HISTORY OF THE COMMON MARINE SHRIMP, PENAEUS SETIFERUS (LINNAEUS)

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The purpose of this report is to assemble information concerning the early stages in the life history of the shrimp, *Penaeus setiferus* (Linnaeus), which supports the most valuable commercial fishery of the South Atlantic and Gulf of Mexico regions of the United States.

P. setiferus is an organism of high reproductive potential. A count made by the authors on the ripe ovaries of a female, 172 mm. total length with spermatophore attached, revealed a total of approximately 860,000 eggs. Burkenroad (1934) states that the ovary of a large shrimp may contain 500,000 eggs. Heldt (1938) counted about 800,000 eggs in the ovaries of *P. trisulcatus*, a European species of similar size and closely related to *P. setiferus*. It may be expected, therefore, that a female will produce from 500,000 to 1,000,000 eggs in a single spawning.

Description of the Egg

The size of the ripe ovarian egg has been given by Weymouth, Lindner and Anderson (1933) as ranging from 0.25 to 0.33 mm. in diameter with an average of 0.277 mm. Burkenroad (1934) believed the egg to be about two-thirds this size, or about 0.185 mm. Later he changed this estimate to "—about 0.25 mm. or less," (Burkenroad, 1939). Pearson (1935), through the use of a plankton net, secured nauplius-bearing eggs varying in diameter from 0.38 to 0.42 mm. Later he reports (Pearson, 1939) that the diameter of twenty-five live eggs, also secured with a plankton net, uniformly measured 0.28 mm. Gutsell (1936) obtained measurements ranging from 0.192 to 0.300 mm. on ripe eggs from a female with spermatophore attached. He found that fresh oocytes dissected out in sea water were about 0.30 by 0.36 mm. Much of the variation in the data cited may be due to varying age of the eggs and varying manner in which they were handled (some were measured fresh, others after fixation).

According to Pearson (1939) "the egg of *P. setiferus* is demersal and sinks promptly in still sea water." It is "non-adhesive and spherical" and "possesses a thin transparent membrane, or chorion, that in living and preserved eggs shows a characteristic purplish-blue color in reflected light under the microscope."

Larval Development

Our knowledge of the larval development of *P. setiferus* depends largely on the work of Pearson (1939). To quote from the summary of his paper: "The larval development of *Penaeus setiferus*, the common commercial southern shrimp, consists of ten distinct stages excluding the demersal spherical egg. These stages

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are made up of five forms generally included under the name of nauplius, three forms included under the name of protozoea, and two forms included under the name of mysis." In addition to these ten larval forms, Pearson describes two post-larval stages which precede the true adult form.

In brief, the larval development of *P. setiferus* requires from two to three weeks. Some twenty to twenty-four hours after the egg is spawned the nauplius breaks the chorionic membrane and emerges. Its ovoid body, of 0.30 to 0.34 mm. in length, bears a single, simple eye and three pairs of oar-like appendages which are to become eventually the first and second antennae and the mandibles. Although this minute organism is to a great extent at the mercy of the prevailing currents, it is capable of some movement. In the next twenty-four to thirty-six hours the nauplius undergoes five successive molts to become a protozoea of approximately 1 mm. in length. It now has seven pairs of appendages, a pair of sessile compound eyes in addition to the ocellus, and a complete alimentary tract consisting of mouth, esophagus, stomach, intestine, and anus. Prior to this stage the food of the nauplius has been the yolk material carried over from the egg. This food supply is now exhausted and henceforth the protozoea must capture its own food if it is to survive. This transitional period is without doubt a critical one in the animal's life history.

The third protozoea stage is followed by the first mysis, which is about 3.5 mm. in length, possesses fourteen pairs of functional appendages, and on the abdomen five pairs of buds which will soon become the pleopods. In the second mysis the pleopods are well developed, and rudimentary gills have made their appearance on the thoracic somites. With the succeeding molt the organism ends its larval phase and assumes the general proportions of a miniature adult. At the end of two post-larval stages and fifteen to twenty days after hatching, the young shrimp is only 5 to 6 mm. in length and is still planktonic. During this period of early development, the young shrimp have moved from the saline offshore spawning area to the brackish inside marshes, bays, and estuaries (Weymouth, Lindner and Anderson, 1933). Upon reaching these "nursery grounds" they adopt for the first time, it is believed, a benthic existence.

The factors responsible for this inshore movement of larval and post-larval *P. setiferus* have not been determined. We believe, however, that for the young to reach the nursery grounds they must encounter a favorable incoming current. While capable of some movement, and perhaps responsive to a salinity gradient, they would certainly be quite helpless against out-going currents. *P. setiferus* has a long spawning season, which in Louisiana extends from March to September; consequently, at intervals during this period the young are bound to encounter favorable conditions for their inshore migration.

Although spawning usually takes place offshore, schools of adult shrimp have been known to approach the coast and spawn close to inlets. When such a spawning occurs, the eggs may be swept through the passes on incoming currents, and the larvae may reach the nursery grounds within a few hours.

Young Shrimp

As stated above and previously reported by Weymouth, Lindner, and Anderson (1933), young shrimp approximately 7 mm. in length are found during the early

spring months in the brackish inside areas which serve as their nursery grounds for the next four to eight weeks of their existence. This habitat is a rich feeding ground characterized by shallow water, muddy bottoms, rather widely fluctuating seasonal temperatures, and moderate to low salinity. Numerous seine and frame-net collections in these areas have yielded quantities of small shrimp 7 to 10 mm. in length; whereas frequent hauls with the same gear during the same period along the ocean and Gulf beaches have failed to yield any *P. setiferus* of this size, although other species of shrimp were found.

As the young grow, they move from the shallow waters of marsh, bayou, and lagoon into the deeper creeks, rivers, and bays, making their first appearance on the inside fishing grounds when about 50 mm. long. In Louisiana the lower limit of the size distributions obtained from operations with commercial gear in the fishery was 43 mm., in Georgia 58 mm., and in Texas 63 mm.

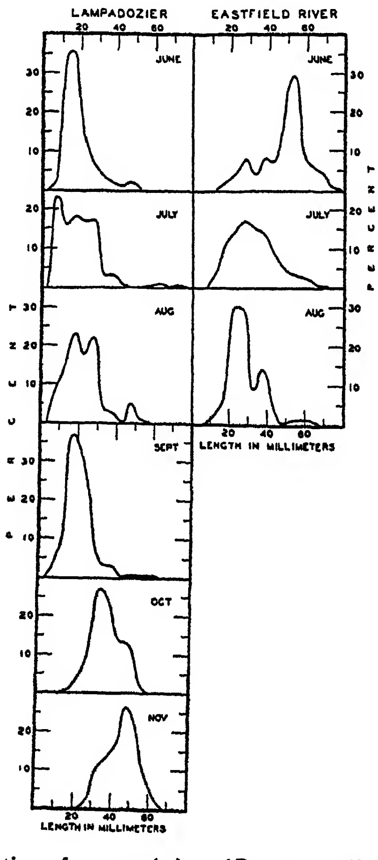

FIGURE 1. Size distribution of young shrimp (*Penaeus setiferus*) seined from the nursery ground areas of Lampadozier and Eastfield River, Georgia. Males and females combined. For the Lampadozier series the curves are based on 200 shrimp in June, 200 in July, 100 in August, 200 in September, 100 in October, and 92 in November. The Eastfield River series are based on 100 shrimp in June, 400 in July, and 100 in August.

To illustrate the population on a typical nursery ground area, the length frequency distributions of small shrimp from seining and frame-net operations in two localities in Georgia are shown in Figure 1. The Lampadozier series, which covers a period from June to November, was obtained entirely by seining in one particular locality in a section representing the inner reaches of the nursery grounds. The young shrimp of the Eastfield River series were somewhat larger in size; they represent collections (covering a period from June to August) taken with both seines and frame-nets in an area midway between the upper nursery grounds (represented by the Lampadozier series) and the lower bays or sounds. The apparent reverse order in the sizes of shrimp in the Eastfield River series is due, it is believed, to the exodus late in June of the larger shrimp, a product of an early spawning, and the entrance in great abundance into the River in July and August of the young from the peak spawning period of May and June.

In the Lampadozier section during June and July the average length of the shrimp was 18 mm. with a range from 8 to 48 mm., although in July a few scattered longer individuals were obtained. In August the average length had increased to 23 mm. with a range from 8 to 53 mm. During September the average length was maintained at 23 mm. with the bulk of the population ranging between 8 and 38 mm., although scattered individuals up to 78 mm. in length were secured. By October the average length had increased to 38 mm. with a range from 18 to 53 mm. During November the average length rose to 48 mm. with a range of 28 to 63 mm.

From the Lampadozier data, the increase in the lower limits of the length frequency distribution from 8 mm. in September to 18 mm. in October and to 28 mm. by November, indicates that after September no new recruits were appearing on the nursery grounds. September marks the end of the spawning season in Georgia (Anderson, Lindner and King, 1948).

Relationship of Nursery Grounds to Commercial Catch

The distribution of the shrimp fishery in itself obviously indicates that passes and the adjacent inland waters are of prime importance to the species. Louisiana, which has a combination of more passes and a vastly larger inland water area landward of these passes than any other state, produces about two-thirds of the shrimp caught each year throughout the entire South Atlantic and Gulf region. Likewise, Georgia and South Carolina, whose shorelines have the most numerous passes and favorable inside waters on the South Atlantic Coast, develop the greatest numbers of shrimp in that section. As a consequence, we conclude that the number of openings to the outside waters and the extent of favorable nursery grounds are two of the major physical factors influencing the production of shrimp in the various sections of the fishery.

In addition to the number of passes and the area of nursery grounds, a coastal or nearby offshore area of relatively shallow water, high salinity, and mud or clay bottom also seems to be a requisite. The Florida peninsula between Fort Pierce on the east coast around almost to St. Marks on the west coast lacks this and likewise lacks shrimp. It is not yet known whether this factor is a requirement of adults or larvae or of both.

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MITOCHONDRIAL ARRANGEMENT IN ALVEOLAR EPICYTES AND FOAM CELLS OF MOUSE LUNGS, PARTICULARLY AS INDUCED BY THE VACUOLOIDS[1]

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INTRODUCTION

The problem of the causation of the design taken collectively by the mitochondria in a living or a fixed and stained cell has interested cytologists for many years. For instance, Lewis and Lewis (1915, p. 352), in studying the living cells of tissue cultures, ask: "What is it that governs the arrangement of the mitochondria? Is it the shape of the cell, the influence of the central body or of the nucleus, the internal structure of the cytoplasm, or do the metabolic activities of the cell govern the size, shape and arrangement of the mitochondria?" Full answers to these important questions have not yet been forthcoming, and the problem is a complicated one. No doubt each of the factors mentioned plays a part.

It seems clear, too, that the physical influence of mechanically inert bodies in the cytoplasm is a formative factor which may be predominant, for mitochondria occupy the general cytoplasm and not the special masses, living or dead, which may find a place in it; and hence anything which molds this general cytoplasm will incidentally establish the morphological pattern of the mitochondria in it. Thus Cowdry (1914) found that the mitochondria of large spinal ganglion cells occurred between the flakes of Nissl substance, and Thurlow (1917) observed that in nerve cells of the cranial nuclei the mitochondria avoided the canalicular apparatus. Similarly the Lewises (1915) showed, in their Figure 26, the mitochondria arranged in a network around the fat droplets of a tissue culture cell. Foreign body inclusions act likewise. This simple mechanical influence on mitochondrial arrangement is herein shown to be exerted by vacuoloids in the pulmonic alveolar epicytes and foam cells to such a degree that the ensuing picture is outstanding and characteristic.

Alveolar epicytes are the residual epithelial cells in the pulmonary alveolar walls (Macklin, 1946). They are also called "septal cells," "niche cells" and other names. Although in the marginal alveolar bases (Macklin, 1945) and other places they have but one air face and rest upon connective tissue, in the interalveolar partitions they frequently have two air faces. These are often of unequal size, the larger overlying the head of the cell and the smaller the foot. In silverwashed material the head and foot are each encircled by a line of silverized material which is part of the silver lineation of the alveolar walls and bronchioles (Macklin, 1938). At alveolar wall intersections the epicytes, as herein discussed, not seldom have three air faces (Fig. 5). This trifaciality is like that found in certain dust cells of mouse lungs

[1] A grant in aid of this investigation by the National Research Council of Canada is gratefully acknowledged.

treated with ammoniacal silver solution (Macklin, 1948). Epicytes may assume a phagocytic rôle (Macklin, 1946). Other functions have been ascribed to them (Hayek, 1942; Sjöstrand and Sjöstrand, 1938). That they may, on occasion, become malignant, so initiating a primary cancer of the lung, has been admitted as a possibility (Macklin, 1938). Their most prominent and characteristic feature is an array of vacuoloids which occupies much if not most of the cytoplasm (Macklin, 1947a). These are clear, round, discrete, non-lipoidal bodies averaging 0.5 μ to 0.75 μ in diameter, which do not take stains (Brodersen, 1933). They are relatively stable, indenting the nucleus in fixed and stained sections.

Alveolar foam cells of mammalian lungs have been described with special reference to their vacuoloids (Macklin, 1947b). They are found in ordinary histological sections, and some of them may be recovered by what has been termed the "gash-irrigation" technic, in which the fresh, collapsed lung is incised through a drop of physiological saline solution and the preparation inverted over a glass slide on which is received the emerging fluid carrying loose cells from the peripheral alveoli. This fluid is then spread and stained as for blood. Foam cells are regarded as originating from epicytes and possibly also from the diversifying epithelium of the bronchioles at the marginal zone adjoining the alveolar ducts, and as being developmental brothers of the dust cells. Difficulty may be encountered in distinguishing the smaller foam cells from well developed epicytes, in sections. The alveolar foam cells are thus of entodermal origin, and are not to be confused with the mesodermal "foam cells" of the pathological literature. As the name implies, they have a foamy appearance, the numerous vacuoloids accounting for the clear spots, which in sections often misleadingly seem to be merged. In dry smears the vacuoloid diameter may reach 1.5 μ.

There is no reason to suspect that the mitochondrial arrangements hereinafter described are peculiar to the alveolar epicytes and foam cells of the mouse. They are probably to be found in these cells throughout the mammalian class at least.

Material and Method

This short study is upon albino mouse lungs freed from as much blood as possible by hemorrhage and moderately distended by the prompt intratracheal injection of Regaud's fixing fluid. Paraffin sections, stained by Bensley's adaptation of the Altmann technic (Cowdry, 1943), reveal the mitochondria in brilliant ruby-red. Most of the mitochondria in epicytes and foam cells are of round or oval form. These are seen in all parts of the general cytoplasm. The ovals grade into short thick rods with rounded ends. Filaments, often beaded, occur. Mitochondria differ in size, the largest being conspicuous while the smallest are seen with difficulty. It is possible that the degree of differentiation with picric acid has something to do with the optical impression of size, which seems less in over- than in under-differentiated cases; but this factor can hardly be in operation when mitochondria in the same cell are being considered, for these have presumably been subjected to uniform technical action. It is the impression that the mitochondrial content of the well developed epicytes is more conspicuous than that of the simpler epicytes on the one hand and the larger foam cells on the other. These mature foam cells are lighter in color, lacking the marked rosy hue typical of the mature epicytes, and in them the mitochondria appear generally smaller and more weakly staining than in the smaller foam cells and epicytes, and are predominantly of coccoid form. Thus

there is considerable variation in the relative prominence of the mitochondrial picture in the various cells examined.

PERIVACUOLOIDAL GROUPING OF MITOCHONDRIA

The design of groups of mitochondria in typical epicytes and foam cells, as seen in well stained thin sections, is dominated by the presence and spacial disposition of the vacuoloids and appears characteristically as a round-meshed sieve with circles of mitochondria, the perivacuoloidal clusters, bounding these clear spheroidal bodies. Mitochondria are never admitted to the interiors of the vacuoloids.

In Figure 2 is shown one of the smaller bifacial epicytes in which the structure is relatively simple, the vacuoloids being in only one layer. Perivacuoloidal groups *p v g* surround these bodies. Those mitochondria which lie just beneath the cell membrane may take part as well in the formation of the inframembranal group *i m g*, and similarly those which are immediately around the nucleus participate as well in the makeup of the perinucleal group *p n g*. This is a good example of an epicyte which goes right through the alveolar wall, and it shows a larger end, the head, well filled with vacuoloids, and a comparatively narrow shank or trunk with a small foot.

Where greater numbers of vacuoloids occur, the appearance is more complex. In the epicyte seen in Figure 3, there are many perivacuoloidal groups, mainly of ovals and spheroids. This cell appears lodged in a crotch of alveolar walls. It has a free air-face above, another to the left and a third to the right, represented in the small foot at the end of the narrow shank which is within a space of the alveolar wall like a pore. Above are rods just beneath and parallel with the free face *i m g*.

In the larger epicytes and foam cells the vacuoloids are in several layers, and hence the perivacuoloidal mitochondria comprise relatively much more of the total content. Figure 7 gives a good impression of this sievelike pattern. It is from a very thin slice of a foam cell at the side of the nucleus. The inframembranal layer is incomplete. In thick sections one can focus up and down through the numerous vacuoloids and find impressive numbers of mitochondria in the cytoplasm around them.

Figures 8, 9 and 10 show this perivacuoloidal arrangement in other foam cells. Under the oil immersion lens the overall picture is uniquely beautiful, and once seen is not forgotten. It is like chains of brilliant rubies festooned about large luminous pearls. Photographs at best give an inadequate representation. The numerous and often large mitochondria are mainly round or oval, and most of them are about the vacuoloids, with an incomplete layer under the cell membrane and another over the nuclear membrane.

Sometimes epicytes which appear to be underdeveloped are found, showing relatively few vacuoloids or mitochondria. In Figure 1, for instance, there is a single row of vacuoloids present only on the air surface. But one mitochondrion appears on the side next to the air, and it is between two vaculoids. This is an example of a very simple distribution of mitochondria. They are massed above and to the right, in the cytoplasm of the surface which rests on connective tissue.

It does not appear that the mitochondria are attracted by the vacuoloids, but rather that they occupy inertly the available space around them. By no means all of the vacuoloidal surface is contiguous to mitochondria. Most of the mitochondria around the vacuoloids are of the spheroid or ovoid type; but there

PLATE I

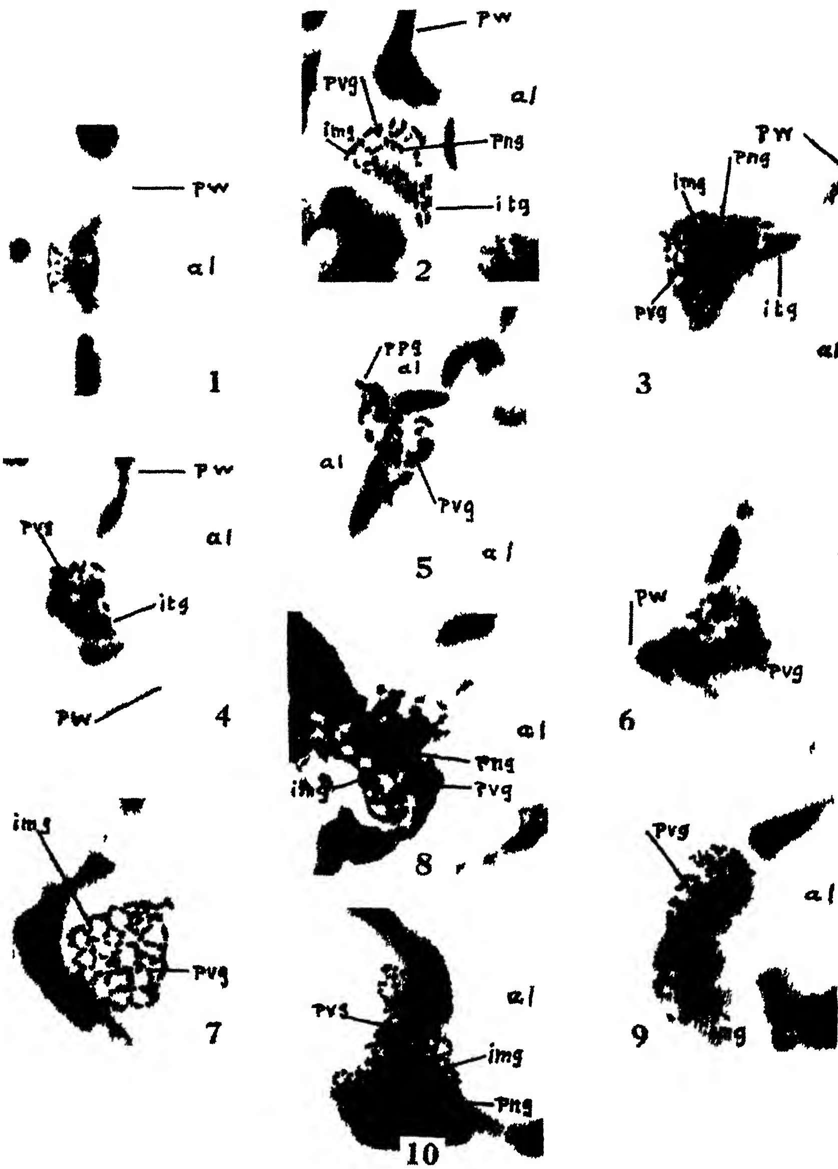

PLATE I

The ten figures are photomicrographs at 1900 diameters made with a Bausch and Lomb 1.9 mm. 1.32 N.A. oil immersion fluorite system objective and 10 × ocular from 3 μ Altmann sections of mouse lungs prepared as described. On the prints the mitochondria were intensified with India ink applied with a fine pen on consultation of the original cell under the oil immersion lens. The first six figures are regarded as epicytes and the remainder as foam cells. Details of mitochondrial arrangement are given in the text.

are many short rods and these are found with the long axis lying tangentially to the vacuoloidal surface. There is no reason to suggest that mitochondria are in any way concerned in the formation of the vacuoloids or that they are influenced in form, size or any other way by contiguity with the vacuoloids. Experimental swelling and distortion of the vacuoloids is reflected in spreading and attenuation of mitochondrial arrangements around them.

Arrangements Not Determined by the Vacuoloids

When epicytes are so cut as to show the long axis of the cell approximately parallel with the optical plane, we may see mitochondrial rods of the intratruncal group *i t g* lying more or less parallel with one another in the trunk or shank and reaching to the foot (Figs. 2, 3). This region of the epicyte lies within the alveolar wall close to the capillaries. In cross sections of such shanks the now dotlike mitochondria are disposed in a circle. This arrangement suggests the shrunken staves of an empty barrel. When cut at a slant such a group appears as in Figure 4. No reason for this peculiar pattern is apparent.

Mitochondria have been noted in epicytes and foam cells lying close to the cell membrane *i m g*. As rods and filaments they often lie parallel with this membrane, and sometimes occur in a double row (Fig. 9). Another layer, which may be indefinite and typically composed of shorter forms, has been noted in the perinucleal cytoplasm *p n g* (Figs. 3, 8, 10). In the edges of the heads of epicytes, where the inframembranal cytoplasm underlying the air surface merges with that adjoining the connective tissue surface, the mitochondria of the peripheral group *p p g* (Fig. 5) in lateral view may have a curious pointed appearance like a pile of sticks, as in the profile of the supports of a North American Indian wigwam, which is difficult to photograph; while other groups simulate the downdrooping branches of the tops of balsam trees. Such clusters contain rounded and oval forms as well as rods. Sometimes mitochondria in these edges are packed in a dense triangular mass. Again, no explanation for these curious formations has been found.

Epicytes on marginal alveolar walls (those which rest on connective tissue) are well endowed with mitochondria. One of these is represented in Figure 6 in the angle between adjoining alveoli. To the left, a group of mitochondria juts into the partition *p w* separating the upper from the lower alveolar space. Perivacuoloidal formations are seen here as in other epicytes.

Substantially the same representation of mitochondrial arrangement in these cells was obtained after the use of Bensley's acid violet-safranin O (Bensley, 1911; Lillie, 1948, p. 98), and Regaud's modification of the iron hematoxylin method of Heidenhain (Cowdry, 1918), though hitherto, in the author's hands, less brilliantly.

Abbreviations:

a l	alveolus,
i m g	inframembranal group of mitochondria,
i t g	intratruncal group,
m w	marginal alveolar wall,
p n g	perinucleal group,
p p g	peripheral group,
p v g	perivacuoloidal group,
p w	partitional alveolar wall.

Summary

In epicytes and foam cells the combined perivacuoloidal groups of mitochondria present, in thin sections, an outstanding and characteristic lacelike picture based on the disposition and condition of the vacuoloids. Its determination is probably mechanical. Mitochondria are never found within the vacuoloids. An incomplete layer immediately underneath the cell membrane, and another around the nucleus, are found. In the former the rods often lie parallel with the membrane and sometimes in double rows.

In epicytes there is often a distinctive group, mostly of rods, which suggest the outer layer of the fasces, and lie in the long axis of the shank, arranged in a circle around a central area devoid of them. Bizarre angulated and branched arrangements are noted in the peripheries of the heads of epicytes.

In foam cells the mitochondrial content varies, often being abundant and conspicuous, and again, perhaps in older cells, relatively inconspicuous.

Acknowledgment

I wish to thank Mr. Charles E. Jarvis for his excellent work in the preparation of the sections and photomicrographs.

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THE CHARACEAE OF THE WOODS HOLE REGION, MASSACHUSETTS

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Introduction

It is extremely doubtful that any other comparable area in North America has been subjected to such constant investigation for fresh-water algae over such a long period of time as has the region in the vicinity of Woods Hole, Massachusetts. Specimens which have become widely distributed in herbaria in this country and abroad show that an almost continuous series of collections has been made since the days of Agassiz and the establishment of the original marine biological laboratory on Penikese Island. Among these collections, many specimens of Characeae have been accumulated by generation after generation of students and investigators, until today one finds an abundance of herbarium material available for study. The objectives of the present paper are primarily to present a workable local flora for the Characeae for use of students of phycology and the independent investigator who employs these plants in his research; and secondarily to provide a systematic treatment of the local species based upon a study of available specimens.

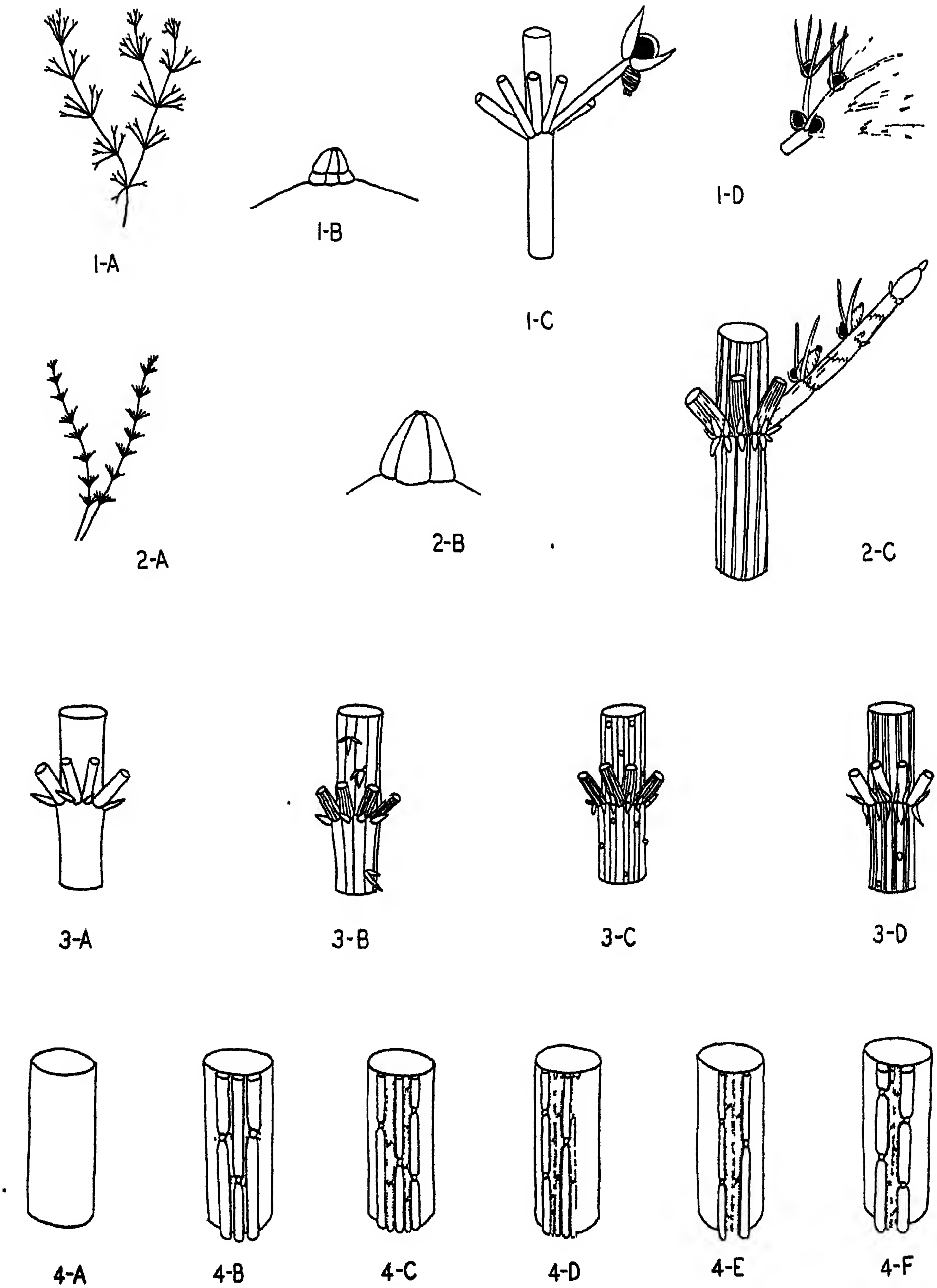
During the summer of 1947, the writer undertook a survey of all accessible bodies of fresh and brackish water in the Woods Hole region. Specimens of Characeae were collected from all places where found, and herbarium mounts and preserved material were prepared. Duplicates of representative forms have been distributed to the New York Botanical Garden, to the herbarium of the Marine Biological Laboratory at Woods Hole, and to certain private herbaria.

The Woods Hole region as delimited for the present paper is approximately the same as that covered by Croasdale (1935) with the addition of certain islands. Roughly, the area includes the islands of Nantucket and Martha's Vineyard, the Elizabeth Islands, and the southern tip of Cape Cod. Names of localities and ponds (cf. Table I) follow those used by Croasdale. Ponds in areas not included by that writer and for which the local names are not known to the present writer are assigned local names as indicated on herbarium labels.

The ponds of the region vary greatly as ecological habitats. In general, the isolated inland ponds result from the glacial knob-and-kettle topography, and tend to be slightly to strongly acid. The coastal ponds are largely lagoons which have become partly or totally isolated from the ocean by barrier beaches or bars. These tend to be markedly alkaline, and salinity varies from strictly marine to weakly brackish. These differences are reflected in the quite distinct characean flora of the two types of ponds. Correlations of distribution and ecological variables will be reported elsewhere.

Despite the wide range of aquatic habitats, the characean flora of the Woods Hole region is not great. With the possible exception of *Nitella Morongii* Allen, all the species reported also occur on the mainland. On the contrary, a number of

PLATE I



species are lacking which could well be expected in the region studied, because of their frequent occurrence on the adjacent mainland. Among those noticeable by their absence are *Chara contraria* Kutz., *C. vulgaris* L., *C. sejuncta* Braun, *C. gymnopitys* Braun (= *C. fibrosa* Ag. *ex* Bruz.),[1] *C. fragilis* Desv. *ap.* Lois. (= *C. globularis* Thuillier), *N. opaca* (C. Agardh *ex* Bruz.) C. Ag., *N. acuminata* Braun *ex* Wallm., *N. capillaris* (Krocker) Groves & B.-W., *N. tenuissima* (Desv.) Kutz., and *Tolypella* spp.

In addition to the specimens obtained during this survey, the writer has had the opportunity to inspect material from the region in various herbaria, including the following: Brown University (BRU)—courtesy of Dr. W. H. Snell; Chicago Natural History Museum (F)—courtesy of Dr. F. Drouet; Farlow Reference Library and Herbarium (FH)—courtesy of Dr. W. L. White; Marine Biological Laboratory (MBL); Maria Mitchell Association of Nantucket (MMA)—courtesy of Grace Wyatt; New York Botanical Garden (NY)—courtesy of Dr. D. P. Rogers, Dr. F. J. Seaver, and Rosalie Weikert; University of California (UC)—courtesy of Dr. H. L. Mason and Dr. G. F. Papenfuss; Yale University (Y); and the private herbaria of E. T. Moul (ETM), Dr. M. S. Doty (MSD), and Dr. W. R. Taylor (WRT). The abbreviations indicated for these herbaria and employed throughout the text follow Lanjouw (1939) with the exception of the writer's herbarium (RDW), other private herbaria (ETM, MSD, WRT), and those for which no standard abbreviation is listed (MBL, MMA).

The literature of the Woods Hole Characeae consists of a few scattered papers. Halsted (1879) reported *N. gracilis* (Sm.) Ag. from Nobska Pond and *C. coronata* var. *Schweinitzii* Braun from shallow ponds at Woods Hole. T. F. Allen (1880;

[1] Zaneveld (1940: 153).

PLATE I

Diagrams illustrating terminology and certain morphological details of the Characeae.

FIGURE 1. Nitella. Diagnostic features: **1-A**, branchlets in the whorls (once) furcate; two true branches shown. **1-B**, coronula of oogonium with ten cells (6 visible) in two tiers. **1-C**, axial node, without stipulodes; axis ecorticate. **1-D**, one fertile branchlet twice furcate (divided) into four secondary rays at the first furcation and 3–4 tertiary rays (dactyls) at the second furcations; three antheridia shown terminally on rays in whorl of rays of the next order; one oogonium shown lateral at the first branchlet node.

FIGURE 2. Chara. Diagnostic features: **2-A**, branchlets in whorls, not furcate; no true branches. **2-B**, coronula of oogonium with five (3 visible) cells in one tier. **2-C**, axial node with two rows of stipulodes (diplostephanous); axis corticated; branchlet not furcate; antheridia below oogonia at branchlet nodes; two elongated bracteoles shown subtending each oogonium; reduced bracts at sterile branchlet node and at fertile nodes abaxial to bracteoles.

FIGURE 3. Chara. Terminology of axial nodes: **3-A**, ecorticate, haplostephanous (stipulodes in one row). **3-B**, haplostichous, haplostephanous. **3-C**, diplostichous, diplostephanous (stipulodes in two rows). **3-D**, triplostichous, diplostephanous.

FIGURE 4. Chara. Terminology of axial cortication. A few cortical filaments shown on a segment of internode. Primary cortical cells drawn solid, secondary cortical cells dotted. **4-A**, ecorticate (without cortication). **4-B**, haplostichous, secondary cortical cells of each cortical node not elongated. **4-C**, diplostichous, secondary cortical cells about half the length of primary cortical internodal cells, resulting in one row of secondary cells between each row of primary cells. **4-D**, triplostichous, secondary cortical cells about equal in length to the primary cells, resulting in two rows of secondary cortical cells between each row of primary cells. **4-E**, triplostichous, aulacanthous (secondary cortical cells greater in diameter than primary cortical cells). **4-F**, triplostichous, tylacanthous (primary cortical cells greater in diameter than secondary cortical cells).

1882a) reported *C. crinita* f. *leptosperma;* and later (1896) a form near *Nitella minuta* or *N. batrachosperma,* possibly a new species *N. maxceana* Allen, from Nantucket; and the description of *N. Morongii* Allen. Maria Owen (1888) listed the following species from Nantucket: *Nitella batrachosperma, N. flexilis, N. flexilis* var. *subcapitata, N. Morongii, N. mucronata, Chara coronata, C. coronata* var. *Schweinitzii, C. crinita, C crinita* f. *leptosperma, C. fragilis* var. *delicatula.* The most recent work is by Croasdale (1935) who reported *Nitella gracilis* from Wood and Golf Ponds, *N. flexilis* from Cuttyhunk, *N. mucronata* var. *gracillima* from Wood Pond, *Chara canescens* and *C. fragilis* from Chara Pond, and *C. delicatula* from Cuttyhunk. This work included a diagnostic key to the species.

The terminology employed in the present paper follows the majority of recent workers in the field. Because of variations in usage of certain terms in the literature, the writer's usage of each term is defined in the discussions of the class, family and genera. Further, the structures are labeled in Plate I.

The nomenclature is that which, in the writer's opinion, represents the valid name; and where marked deviations from recent monographic treatment exist, reasons for such changes are given. Synonyms listed include only those which occur in recent literature. For more complete synonymies, the reader is referred to Migula (1897), Groves and Bullock-Webster (1920; 1924), Zaneveld (1940), and Wood (1948b) for the genus Nitella in North America.

The descriptions and figures, with exception of illustrative diagrams, were taken from specimens collected in the region, and thus reflect only the characteristics of the local forms. All such specimens are in the writer's herbarium (RDW). It should be noted that the description of each species is based entirely on a single specimen. Variations from this plant are to be found in the discussion of variations following each description.

In addition to the persons and institutions indicated above to whom the writer is deeply indebted for the opportunity to study herbarium material, he is especially indebted to Dr. Hannah Croasdale who guided the writer to many collecting sites, and to Dr. M. S. Doty for his encouragement and aid in the completion of the present work. Most sincere gratitude is due G. O. Allen, Esq., of England, Dr. W. R. Taylor, and Dr. M. S. Doty for timely criticisms of the original manuscript.

The Key

The following key is intended to be strictly analytical in nature, and employs vegetative characteristics as far as possible. The most convenient characteristics are listed first in each entry. The key disregards the distinctions between genera. A differential synopsis of tribes and genera may be found under "Family Characeae."

1a. Main axes uncorticated 2
1b. Main axes corticated 6
  2a. Branchlet whorls not subtended by stipulodes; branchlets one or more times furcate (forked): antheridia born terminally on rays; oogonia borne laterally at furcations of branchlets, thus situated below antheridia 3
  2b. Branchlet whorls subtended by one series of stipulodes; branchlets not furcate; antheridia borne laterally at branchlet nodes; oogonia above antheridia ....5. *Chara Braunii,* p. 193

3a. Ultimate rays (dactyls) of branchlets not terminated by a small pointed cell (mucro) ....1. *Nitella flexilis,* p. 187
3b. Ultimate rays of branchlets terminated by one or more such small cells 4
4a. At least some of the dactyls more than two-celled, the tips varying from a single-celled mucro to a tip with two or more such mucros; oogonia geminate (two at a node) to aggregate (more than two at a node), but occasionally solitary; plants large and robust, main axes in mature plants generally exceeding 400 μ in diameter.
2. *Nitella megacarpa,* p. 188
4b. Dactyls strictly two-celled including the small terminal mucronate cell; oogonia solitary (one oogonium at a fertile node); plant distinctly small and delicate, main axes generally less than 350 μ in diameter 5
5a. Branchlets once or twice furcate; fertile whorls reduced to small heads; oospore membrane appearing roughened, but not reticulate.
3. *Nitella Morongii,* p. 189
5b. Branchlets 3 or more times furcate; fertile whorls not reduced to heads; oospore membrane reticulate ....4. *Nitella transilis,* p. 191
6a. Spines (at least some) on main axes generally in groups of 2–5 (fascicled); cortication haplostichous (number of corticating cells equal to the number of branchlets in an adjacent whorl).
6. *Chara canescens,* p. 196
6b. Spines (if present) on main axes not fascicled (very rarely paired), solitary; cortication of the main axis appearing diplostichous to triplostichous (corticating cells 2–3 times as many as branchlets in adjacent whorl) 7
7a. Plants monoecious, the oogonia and antheridia on same plant, though by loss of one or other of the sex organs they may appear dioecious.
7. *Chara delicatula,* p. 198
7b. Plants dioecious, the oogonia and the antheridia borne on separate plants (thorough inspection of a number of plants must be made before the dioecious condition can be considered proved) ....8. *Chara aspera,* p. 199

Class Charophyceae

More or less bushy, green, submerged, attached, aquatic plants which vary in size from 0.5 cm. to nearly 2 meters in height. Rhizoidal portions hyaline, and ramifying in the soil. Laterals borne in whorls along an erect axis. Certain forms deposit an external incrustation of lime; some become dark brown to black. Both conditions may be uniform or may develop as bands encircling the cells. Habitat strictly fresh water in most species, some species more tolerant of low salinity, and some restricted to brackish water. No marine forms are known.

Vegetative portions fundamentally consisting of a uniaxial filament, the filament exhibiting alternating elongate internodal cells and short nodal cells. The internodal cells merely elongate, but the nodal cells divide three or more times in such a way as to form central nodal cells and a series of peripheral nodal cells. One

or more central nodal cells at each node cut off apical cells, which continue development of filaments (*main axis*). A dominant filament so formed is considered the main axis, others are considered as *branches* (laterals of unlimited growth). The peripheral nodal cells each cut off meristematic initials which form laterals of limited growth—a whorl of *branchlets* at each node. Other peripheral nodal cells (e.g., Chara) cut off meristematic initials which form corticating filaments lying along the outside of the internodal cell This layer is the *cortication*. From other peripheral nodal cells may arise elongated cells, the *stipulodes*, which spread laterally subtending the branchlets (Pl. I, Fig. 3). The branchlets may divide (furcate) into two or more segments or *rays* (Pl. I, Fig. 1–D) at each node to the fourth or fifth order (Nitella), or the branchlets may remain strictly undivided (Chara), and consist of alternating nodes and internodes (except for terminal ecorticated cells). Undivided branchlets produce only one central nodal cell at each node with accompanying peripheral nodal cells. Spine-like processes from these peripheral branchlet nodal cells form *bracts* (Pl. IV, Fig. 4-C) at each branchlet node. The branchlets may be completely or partly corticated or totally ecorticate.

The oogonium or egg-producing cell is fundamentally an apical cell terminal borne on a modified lateral and ensheathed in an encasement of five spirally twisted laterals of limited growth, the *enveloping cells*. At first these are straight, but become tightly spiralled about the oogonium in maturity. The writer employs the term *convolutions* to indicate the resulting spirals apparent on the female gametangium. Each enveloping cell is terminated by one small cell in the tribe Chareae and by two small cells in the tribe Nitelleae, which results in a *coronula* of five cells or ten cells in two superimposed tiers, respectively. The entire female gamentangium is known taxonomically as the *oogonium*.

The oogonia generally arise as laterals of the antheridial stalk (see below) in Chara, or from a peripheral nodal cell of a branchlet in Nitella and species of Chara where gametangia are not conjoined. In the genus Chara, elongated cells, the *bracteoles* which often closely resemble bracts, also develop from the antheridial stalk. The *oospore* develops within the enveloping cells of the oogonium. The impression of the enveloping cells on the oospore results in spiralled ridges, the *striae* or *striations*. The outer membrane of the oospores frequently develops characteristic *oospore membrane patterns*.

The biflagellate, spiral *sperms* are produced singly in cells which occur in filaments (*capitular filaments*) borne terminally on laterals (*manubria*) which project inwardly from the centers of four or eight *shield plates*. The shield plates coalesce to form a spheroid structure, and radiating lines (partial septa [modified cells?]) project from the periphery toward the center of each shield plate. The entire spherical male gametangium is known taxonomically as the *antheridium* (Pl. I, Fig. 1-D). The antheridia arise from peripheral nodal cells of branchlets (Chara) or from the apical cell of a ray (Nitella).

The vegetative plants are haploid; meiosis, as far as is definitely known, occurs in the germination of the zygote. Both monoecious and dioecious species occur.

For a more detailed discussion of the morphology, the reader is referred to Fritsch (1935: 447–465).

FAMILY CHARACEAE

The only existing family with characteristics of the class. The family has been divided into tribes and genera based upon both sexual and vegetative characters. A synopsis to this classification is given below.

Coronula of the oogonium consisting of ten cells in two tiers—Tribe NITELLEAE Ganterer (1847: 8), *pro parte*, *emend.* Leonhardi (1863: 69).

- Antheridia apical on a ray in the furcation of the branchlets—Genus NITELLA C. A. Agardh (1824: xxvii), *pro parte*, *emend.* Braun (1849a, b: 195, 292).
- Antheridia lateral at the furcation of the branchlets—Genus TOLYPELLA (Braun, 1849a: 199) Leonhardi (1863: 72).

Coronula of the oogonium consisting of five cells in one tier—Tribe CHAREAE Leonhardi (1863: 72).

- Stipulodes at the base of the branchlets lacking.
  - Bracts present, one to two at a node—Genus NITELLOPSIS Hy (1889: 397).
  - Bracts absent—Genus PROTOCHARA Womersley and Ophel (1947: 311).
- Stipulodes at the base of the branchlets present, although sometimes rudimentary.
  - Oogonia normally situated below antheridia; axes ecorticate—Genus LAMPROTHAMNIUM Groves (1916: 336), *emend.* Ophel (1947: 322).
  - Oogonia normally lateral with respect to antheridia, an oogonium situated between two antheridia; axes corticate—Genus LYCHNOTHAMNUS (Ruprecht, 1845: 11) *pro parte*, Leonhardi (1863: 72).
  - Oogonia situated above the antheridia; axes corticate or ecorticate—Genus CHARA Vaillant *ex* Linnaeus (1754: 491).

Womersley and Ophel (1947) recently described a new genus *Protochara*. This genus is founded upon *P. australis* Wom. & Oph., a new species described in the same article. *Nitellopsis inflata* Filarszky & Allen was transferred to the new genus in the combination *P. inflata* (Fil. & Allen) Wom. & Oph. Ophel (1947), in another article, emended *Lamprothamnium* Groves, and included *Chara macropogon* Braun in the combination *Lamprothamnium macropogon* (Braun) Ophel. He also suggested removing *L. Hansenii* Sonder to *Chara Hansenii* (Sonder) Ophel. The material and evidence to support these changes have not as yet been examined by the present writer.

Of the six or seven known genera, three occur in North America; namely, Chara, Nitella, and Tolypella. Of these, Chara and Nitella are represented in our region; although Tolypella is known to occur as near as the Finger Lakes of New York, and has been recorded from Vermont.

Genus Nitella

Branches commonly two or more at a node. Branchlets one or more times furcate into two or more *rays* (Pl. I, Fig. 1-D) at each furcation. The ultimate rays of a branchlet beyond the last furcation are known as *dactyls* (Pl. I, Fig. 1-D). In many species a small terminal cell occurs on the dactyl, and is known as a *mucro* (Pl. III, Fig. 2-A). In some species the fertile branchlets are greatly reduced and

Plate II

modified; in others the fertile branchlet whorls and the branch upon which they are borne are reduced and form more or less dense *heads* (Pl. II, Fig. 2). Fertile heads may be enveloped in *mucus*—a hyaline, gelatinous material. An oogonium arises from peripheral nodal cells of a branchlet, thus replacing a ray, and projects laterally below the whorl of rays. An antheridium occurs terminally on a ray amid the whorl of rays of the next order, replacing the apical cell of the ray. Cells of the coronula of the oogonium ten, in two superimposed tiers of five cells each. Oospores are laterally compressed.

In our region, specimens of Nitella can be immediately distinguished from Chara in the field by the obvious character that the branchlets are divided, whereas they are strictly undivided in Chara.

1. **Nitella flexilis** (Linn., *pro parte*) C. A. Agardh, Syst. Alg., p. 124. 1824.
Chara flexilis Linn., Spec. Plant., p. 1157. 1753.
References for the region: *N. flexilis:* Owen (1888:74); Croasdale (1935:94); var. *subcapitata:* Owen *l.c.*

(Plate II, Fig. 1; Plate III, Fig. 1)

Plant monoecious, 20 cm. high, diffuse but robust. *Branchlets* about eight in a whorl, once furcate into two long dactyls (one occasionally lacking). *Dactyls* (1–) 2[2], equal to or exceeding the primary ray in length, terminated by a sharp-pointed mucronate tip (not a distinct cell). *Gametangia* generally aggregate, (1–) 2 (–3) oogonia and an antheridium at each fertile node. *Oogonia* 850–875 μ long by 690–710 μ broad; coronula about 75 μ broad by 36 μ high, ultimately deciduous; convolutions 7. *Oospores* 530–550 μ long by 472–500 μ broad, black; striae apparently 5; membrane smooth. *Antheridia* 340–415 μ (immature; 515–555 μ in *R. D. Wood 2014*) in diameter. (Descr. from *R. D. Wood 2013* (RDW).)

Variations among the local forms result from differences in development of vegetative structures. In size, plants vary up to 35 cm. high. The dactyls vary from short (1/6 length of primary ray) in *Maria Owen 3,* to greatly elongated (longer than primary ray) in *R. D. Wood 2014*. The rays vary from those which are all equal in length and give a distinctly regular appearance to the whorls as in *R. D. Wood 2030*, to those which are very unequal in length and give a ragged appearance to the whorls as in *T. Morong 3*. The branchlets vary from elongate in *R. D. Wood 2014* giving loose whorls, to very short in *T. Morong 2b* giving ap-

[2] G. O. Allen (corresp., 1948) reports that three dactyls occasionally occur in British specimens.

PLATE II

Drawn from the indicated herbarium specimens which are extant in the writer's herbarium. × 0.5.

FIGURE 1. *Nitella flexilis* (L. *pro parte*) C. Agardh. (*2030.*)
FIGURE 2. *Nitella Morongii* Allen *emend.* Wood. (*E. T. Moul 3173.*)
FIGURE 3. *Nitella megacarpa* Allen. (*2060.*)
FIGURE 4. *Nitella transilis* Allen. (*2021.*)
FIGURE 5. *Chara delicatula* C. Agardh. (*2058.*)
FIGURE 6. *Chara canescens* Lois. (*2027.*)
FIGURE 7. *Chara Braunii* Gmelin. (*2004.*)

pearance of reduced clumps isolated on elongated internodes. The fertile whorls are generally similar to the sterile whorls, but in *W. R. Taylor* (*17085* WRT) the heavily fertile whorls are somewhat reduced into loose heads. The color of dried specimens is generally translucent greenish brown, but certain specimens become opaque dark brown as in *T. Morong 1* and thus suggest *N. opaca* (but our specimens are monoecious!). These extreme characteristics appear to occur in random combination, and could permit designation of easily ten or more different forms, but none of the combinations seem sufficiently constant to merit assignment to varietal status. Allen (1871: 9; 1880: 11) and Owen (1888: 74) have reported those forms in which fertile whorls are somewhat reduced as var. *subcapitata* (Hartm.) Groves (= var. *nidifica* Groves, H. & J.), and Allen (1880: pl. 5) assigned forms with extremely shortened branchlets and stout internodes to var. *crassa* Braun and to form *superne brachyphylla* (in herb. NY).

N. flexilis is easily distinguished in the field, as it is the sole species in our local flora in which the branchlets are only once divided. However, occasional sterile branchlets may not be furcate, and inspection of the fertile whorls may be necessary to demonstrate this feature.

Illustrations: Allen (1892: pl. 6, 6a, 6b); Groves & Bullock-Webster (1920: pl. 8); Migula (1897: 133; 1925: 214, fig. 4, 5); Woods (1894: pl. 26).

Exsiccatae: Phyc. Boreali-Amer., No. *1435, 1691;* Char. Amer. Exsicc., No. *28, 29, 30.*

Localities: CAPE COD: Weeks Pond—*R. D. Wood 2016*, July 26, 1947 (RDW); CUTTYHUNK: Clubhouse Pond—*R. D. Wood 2030*, July 31, 1947 (RDW, NY); Sheep Pond—*W. R. Taylor*, June 28, 1932 (*17085* WRT, NY); Sheep Spring—*W. R. Taylor*, June 27, 1933 (*16981, 17010* WRT); July 3, 1934 (*16769* WRT); MARTHA'S VINEYARD: Chilmark Pond—*R. D. Wood 2014*, July 20, 1947 (RDW, NY); Tiasquam Dam—*R. D. Wood 2013*, July 20, 1947 (RDW); NANTUCKET: Cato's Pond—*T. Morong 3*, July 22, 1887 (NY); New Lane Pond, pond west of New Lane and S. of Grove Lane—*Grace Wyatt*, Sept. 4, 1947 (RDW); Polpis—*Mary Owen 3*, July, 1879 (NY), as var. *subcapitata;* R. R. Track Pond, pool E. of town [Nantucket] near R. R. tracks—*T. Morong 1*, July 16, 1887 (NY); Sesachacha, drainage ditch S.W. of pond—*Grace Wyatt*, Aug. 12, 1947 (RDW); Weweeder Pond—*T. Morong 2b*, July 16, 1887 (NY); July 15, 1887 (NY); NONAMESSET: South Pond—*Hannah Croasdale 55*, June 15, 1935 (MBL, preserved; *2012* RDW); PASQUE: West End Pond—*W. R. Taylor*, July 2, 1941 (*20840* WRT, FH); ? Pond, west end of island—*W. R. Taylor*, July 3, 1940 (*19691* WRT).

2. **Nitella megacarpa** Allen, Characeae Americanae Exsiccatae, No. 3. 1880.

Nitella microcarpa subsp. *megacarpa* (Allen) Nordstedt *apud* Braun, Fragmente einer Monographie der Characeen, p. 73. 1882.

References for the region: *N. mucronata:* Owen (1888: 74 [pl. not seen]); var. *gracillima* Groves & B.-W.: Croasdale (1935: 94).

(Plate II, Fig. 3; Plate III, Fig. 4)

Plant monoecious, 25 cm. high, robust, bright green. *Branchlets* (4–) 5–6 (–7) in a whorl, up to 3.5 cm. long, spreading widely; whorls at apex of main axes partly convergent and forming a rather broad, terminal clump; branchlets bear gametangia throughout the season, 3–4 times furcate into 5 secondary rays, 2–3 tertiary rays, 2–3 quaternary rays, and occasionally 2–4 quinary rays. *Dactyls* 2–3, 2–3 (occasionally 4–more) -celled; ultimate cell a conical mucro 37–74 μ long; basal cell 100–500 μ long; intermediate cells (when present) generally one or two subcylindri-

cal cells a little longer than broad. *Gametangia* borne on branchlets of all whorls, present throughout the season, solitary to aggregated, not enveloped in mucus. *Oogonia* about 710 μ long by 510 μ broad, 1–2 (–3 or more) at a branchlet node; coronula about 25 μ long by 36 μ broad; convolutions 9. *Oospores* about 420 μ long by 370 μ broad, yellow when immature, darkening to deep brown on maturity, often nearly spherical; striae 5–6; membrane strongly reticulate; reticulae 2.2–3.6 μ in diameter, subquadradic, 9–11 across a fossa. *Antheridia* 265–445 μ in diameter. (Descr. from *R. D. Wood 2060* (RDW).)

This large, bright Nitella is by far the most beautiful species in the region, and one which cannot be confused once it has been seen in the field. In size, the plants vary up to nearly 40 cm. high.

Nordstedt (1882) and Zaneveld (1940) included *N. megacarpa* within the limits of *N. microcarpa* Braun. Our form certainly is very similar in most fundamental respects to this species, and is doubtlessly very close phylogenetically. The local form differs in being a much larger plant with much larger gametangia. The greater general size suggests polyploidy from *N. microcarpa*. If this proves to be the case, the erection of a distinct species is almost required. Furthermore, the writer has seen no specimens which exhibit complete serial intergradation between the two forms. Therefore, until intergrades are seen, or until cytological investigations are completed, the writer prefers to consider *N. megacarpa* Allen a distinct species in agreement with G. O. Allen (corresp., 1948).

A characteristic feature of this plant is the manner in which the main axis is easily separated at the nodes. As a result, specimens are commonly collected, especially with plant hooks, which consist of only the terminal clump of whorls of branchlets. These fragments do not give the impression of massiveness characteristic of the plant.

Illustrations: No habit sketches of this species have been published previously.

Exsiccatae: Phyc. Boreali-Amer., Fasc. 32, No. *1588,* Fasc. E, No. *CII;* Char. Amer. Exsicc., No. *3.*

Localities: CAPE COD: Ashumet Pond—*I. F. Lewis,* Sept. 4, 1926 (WRT); Leech Pond—*C. W. Palmer,* July 27, 1936 (*17199* WRT); Summerfield Pond, South—*C. C. Jao,* Sept. 4, 1933 (*16771* WRT); Weeks Pond—*Hannah Croasdale,* July 28, 1947 (*2019* RDW, MBL, MSD, ETM); *R. D. Wood 2031,* Aug. 1, 1947 (RDW), *2060,* Sept. 6, 1947 (RDW); Wood Pond—*W. R. Taylor,* July 2, 1921 (*3668* WRT).

3. **Nitella Morongii** Allen, Bull. Torrey Bot. Club **14**: 214. 1887; *emend.* Wood, Rhodora **51** (602): 16. 1949.

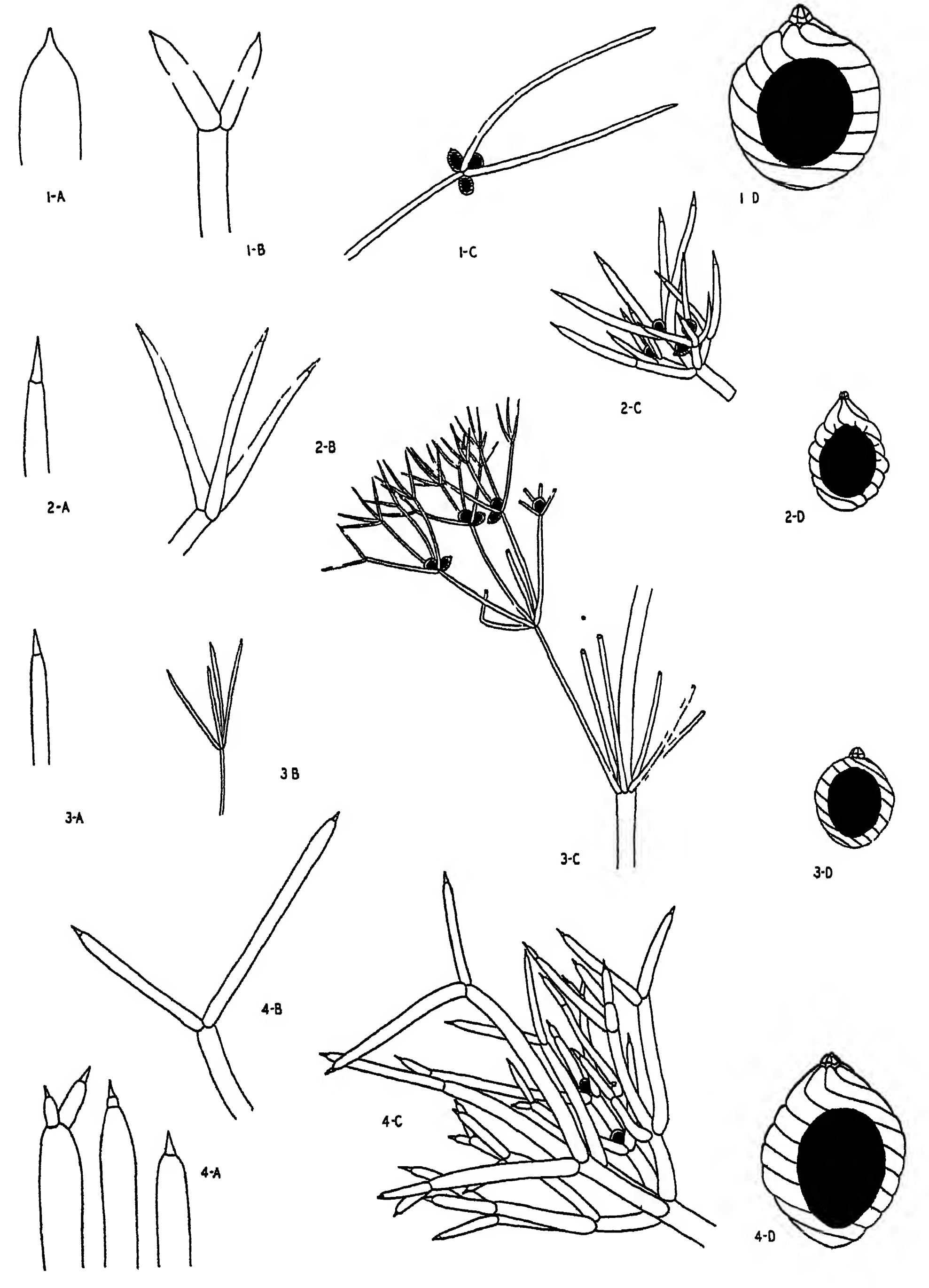
Nitella maxceana Allen, *spec. dub.*, Char. Amer. 2(3): 27. 1896.

References for the region: *N. batrachosperma:* Owen (1888: 74); *N. gracilis:* Halsted (1879: 176), Croasdale (1935: 95); f. *brachyphylla:* Collins *in* Phyc. Boreali-Amer., No. *1195; N. maxceana:* Allen, *l.c.; N. Morongii:* Owen, *l.c.*, Allen, *l.c.*, Wood, *l.c.*

(Plate II, Fig. 2; Plate III, Fig. 2)

Plant monoecious, 14 cm. high, delicate, and with characteristic heads. *Branchlets* of two types, including: (1) the normally expanded sterile or lightly fertile branchlets, and (2) the greatly reduced fertile branchlets. *Sterile branchlets* 2–5 in a whorl, 15–26 mm. long, 1–2 times furcate into 3–5 secondary rays, 2–3 tertiary rays. *Dactyls of sterile branchlets* 2–3, 2-celled, the ultimate cell a conical mucro

PLATE III



which is early deciduous. *Fertile branchlets* 6–7 in a whorl, twice furcate, greatly reduced, 1–3 mm. long; 3–5 such reduced whorls borne on a reduced branch, the entire complex resembling a dense head; heads apparently axillary in sterile whorls or terminal, enveloped in mucus. *Dactyls of fertile branchlets* 2 (–3), 2-celled, one commonly shorter than the other, terminated by an elongated mucro. *Gametangia* solitary, an oogonium and an antheridium at all fertile branchlet nodes, enveloped in a weak mucus.[3] *Oogonia* 290–386 μ long by 210–288 μ broad; coronula 35 × 35 μ. *Oospores* 238–268 μ long by 180–210 μ broad; striae of 5 prominent ridges; membrane roughened with anastomosing lines, almost appearing very finely reticulate. *Antheridia* somewhat flattened longitudinally, 134–148 μ long by 174–179 μ broad, short stipitate. (Descr. from *E. Moul 3173* (RDW).)

Specimens which have been collected since 1888 are quite consistently of a diffuse form as in *E. Moul 3173,* described above. A compact form as in the TYPE COLLECTION *T. Morong,* July 21, 1887, has very short branchlet rays, more fertile whorls at a fertile branch, and early deciduous sterile branchlets. This modification is thought to be the result of extreme ecological conditions exerted by constant trampling by the feet of animals and the muddy water (cf. Wood, 1949). In size, some plants reach 20 cm. high.

Illustrations: Allen (1894: pl. 16), excellent for the original material, but not as emended.
Exsiccatae: Phyc. Boreali-Amer., No. *1195* as *N. gracilis, 1382.*
Localities: CAPE COD: Golf Pond—*G. M. Gray,* July, 1931 (*17006* WRT); *W. R. Taylor,* July 6, 1917 (*2293* WRT); *Hannah Croasdale,* June 24, 1935 (*17078* WRT, NY, *950899* F, RDW); Harper Pond—*E. T. Moul 3173,* July 7, 1947 (ETM, RDW, NY); *Urda K. Wood,* July 11, 1947 (*2011* RDW, NY); Nobska Pond—*W. A. Setchell* and *W. J. V. Osterhout 644,* July 15, 1894 (MBL, FH, *315652* UC); *W. A. Setchell,* July 15, 1893 (P. B.-A. No. *1195,* as *N. gracilis,* NY); ? Pond, Woods Hole—[*Coll.?*], 1883 (NY); NANTUCKET: Maxey's Pond —*T. Morong,* July 7, 1887 (NY, Type of *N. maxceana* Allen); Siasconset, small pond south side of old Sconset Road, opposite "Bloomingdale"—*L. L. Dame,* July, 1886 (NY, in one P. B.-A. packet No. *1382*); in a very muddy pool on the roadside near Siasconset—*T. Morong 4,* July 21, 1887 (FH, NY, TYPE COLLECTION); in a small pool near Siasconset—*F. S. Collins,* Aug. 23, 1896 (P. B.-A. No. *1382,* NY, BRU); NAUSHON: Petchett (or Peckett [?]) Pond—*Hannah Croasdale,* July 5, 1946 (MBL, preserved).

4. **Nitella transilis** Allen, Char. Amer. 2(3): 24. 1896.
References for the region: none known.

[3] Allen (1888) in his synopsis enters *N. Morongii* Allen under "heads not enveloped in mucus," but this point is reported with some question later (Allen, 1894: 15).

PLATE III

All figures have been drawn with the aid of a camera lucida from specimens preserved in 2–3 per cent formalin. The indicated specimens are extant in the writer's herbarium.

FIGURE 1. *Nitella flexilis* (*L. pro parte*) C. Agardh. **1-A**, tip of terminal branchlet cell, × 35. **1-B**, dactyls, × 14. **1-C**, fertile branchlet node, one antheridium and two oogonia, × 14. **1-D**, oogonium, × 35. (*2013.*)

FIGURE 2. *Nitella Morongii* Allen *emend.* Wood. **2-A**, tip of dactyl with terminal mucro, × 35. **2-B**, dactyls, × 14. **2-C**, fertile branchlet, × 14. **2-D**, oogonium, × 35. (*Urda K. Wood 2009.*)

FIGURE 3. *Nitella transilis* Allen. **3-A**, tip of dactyl with terminal mucro, × 35. **3-B**, dactyls, × 14. **3-C**, axial node and part of one branchlet, × 14. **3-D**, oogonium, × 35. (A and B from *2015*; C and D from *2059.*)

FIGURE 4. *Nitella megacarpa* Allen. **4-A**, tips of dactyls showing variations in form of mucros, × 35. **4-B**, dactyls, × 14. **4-C**, fertile branchlet (a very small specimen), × 14. **4-D**, oogonium, × 35. (*2019.*)

(Plate II, Fig. 4; Plate III, Fig. 3)

Plant monoecious, 12 cm. high, very fine and delicate. *Branchlets* 6–7 in a whorl (branches occasionally two at a node), 3–4 times furcate into 6 secondary rays, 5 tertiary rays, and 4 (–5) quaternary rays; quinary rays when present 2–3; branchlets usually exceeding the axial internodes in length, primary rays about 3/5 the total length of the branchlet. *Dactyls* 2–3 (–5), 2-celled. *Gametangia* solitary, an antheridium and an oogonium borne on the second and third, rarely at the first, branchlet nodes, enveloped in mucus. *Oogonia* about 309 μ long by 240 μ broad; convolutions 7–8. *Oospores* about 276 μ long by 170 μ broad; striae of 6 ridges; membrane strongly reticulate. reticulae 10–12 across the fossae. *Antheridia* about 178 μ in diameter, stipitate on stalks about 37–59 μ long; markings on shield extending about half way to base of manubrium, irregular. (Descr. from *R. D. Wood 2015* (RDW).)

The local form of this species is very constant, and apparently does not exhibit modifications found in other parts of eastern North America. In size it reaches 16 cm. in height. Some specimens might be confused with *N. Morongii* because of the size and occurrence of mucus.

Whether *N. transilis* Allen is sufficiently distinct to be separated from *N. tenuissima* (Desv.) Kütz. has been discussed (corresp., 1946–1948) with G. O. Allen. The European *N. tenuissima* f. *gracilior* (*L. Chevallier,* Aug. Sept. 1893. *Gallia occidentalis: In stagnis circa "Bazouges"* (*7185* WRT)) approaches *N. transilis* rather closely. Other intergrades of European *N. tenuissima* exhibit diffuse branching resembling *N. transilis.* Further significant information is accumulating in the writer's herbarium in the form of a series of intergrades from various stations in New England. It seems probable that our specimens may well be included under *N. tenuissima.* At present, the characters which seem sufficient to retain it as a separate species from *N. tenuissima* include: (1) the very regular diffuse whorls, (2) the branchlets exceed the internodes in length, (3) the rather frequent occurrence of gametangia at the first branchlet node, and (4) the stipitate antheridia.

Illustrations: Allen (1896: pl. 23).
Exsiccatae: Char. Amer. Exsicc., No. *31, sub. nom. N tenuissima* f. *longifolia elongata.*
Localities: Cape Cod: Ashumet Pond—*M. S. Doty* & *L. Spiegel 7470,* Aug. 17, 1948 (MSD, RDW); John Pond, Mashpee—*G. W. Prescott 5,* Aug. 30, 1937 (*17973* WRT); Weeks Pond—*Hannah Croasdale,* July 14, 1946 (*6615* MSD), July 28, 1947 (*2021* RDW, ETM, MBL, MSD, NY); *R. D. Wood 2015,* July 26, 1947 (RDW, NY); *R. D. Wood 2059,* Sept. 6, 1947 (RDW, mature).

Genus Chara

Branches formed occasionally at nodes. Branchlets not furcate, divided by nodes and internodes into a continuous series of *articulations;* the terminal two or more articulations may lack nodes, and be merely separated by cell walls. Spine-like processes (*bracts,* Pl. IV, Fig. 4-C) arise from peripheral cells of the branchlet nodes, and form a more or less distinct whorl at sterile and fertile nodes. Branchlet articulations corticated, partly corticated, or uncorticated. Main axes corticated or uncorticated (*ecorticate,* Pl. I, Fig. 3-A, 4-A). Cortication varies from *triplostichous* (Pl. I, Fig. 3-D, 4-D) in which the secondary cortical cells

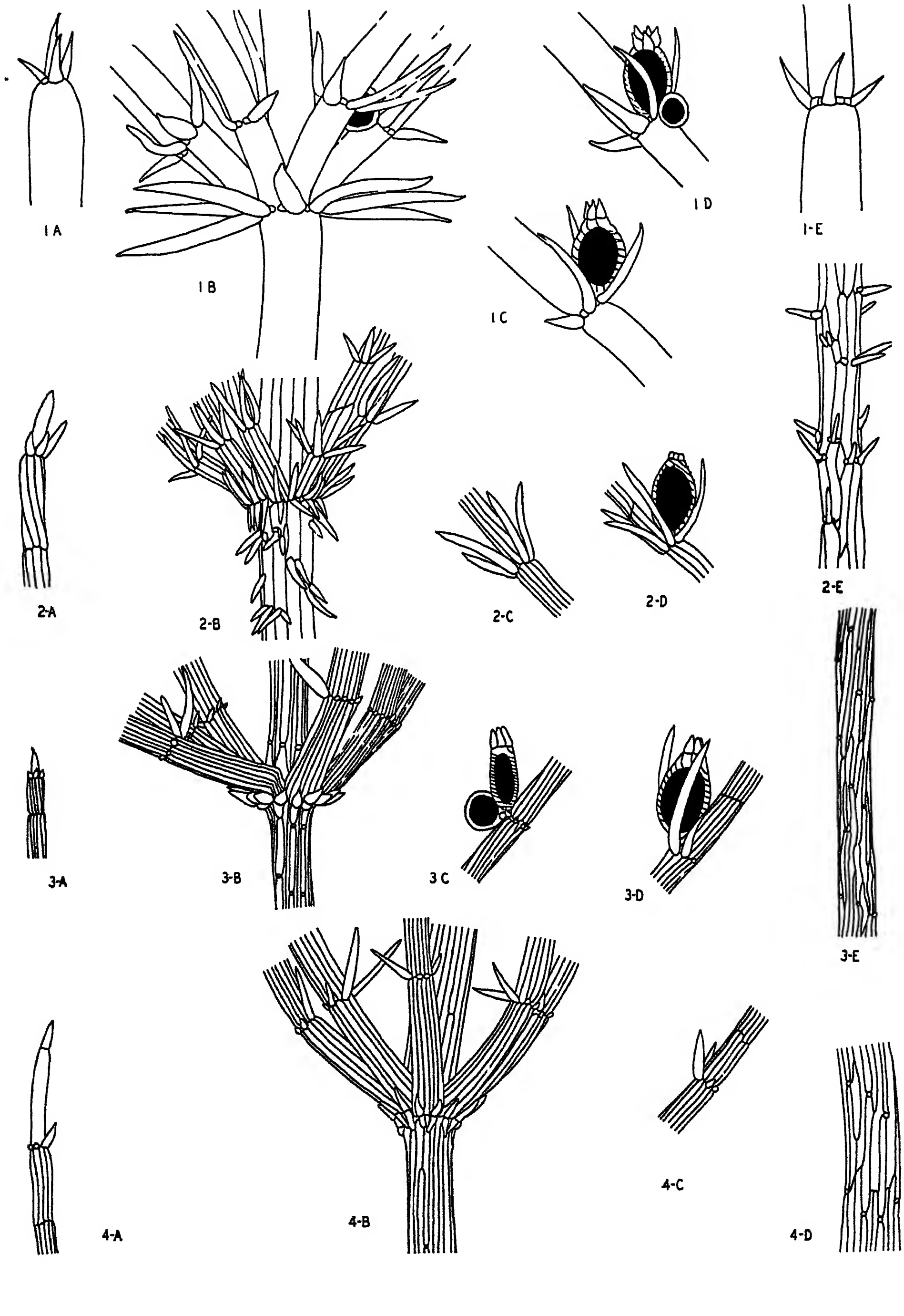
are nearly equal in length to the primary cortical cells, thus forming two series of cells between each primary cortical series (and three times as many cortical filaments as branchlets at an adjacent node); to *diplostichous* (Pl. I, Fig. 3-C, 4-C) in which the secondary cortical cells are about half the length of the primary cortical cells so that the secondary cells from adjacent primary cortical cells series lie end to end forming one continuous series of cells between each primary cortical series (and thus twice as many cortical filaments as branchlets at an adjacent node); to *haplostichous* (Pl. I, Fig. 3-B, 4-B) in which the secondary cortical cells do not become enlarged, thus only the primary cortical cells are apparent in the cortication (and thus the same number of cortical filaments as branchlets at an adjacent node). The details of cortication are best seen by inspecting the cortication of very young axial internodes (*fide* G. O. Allen, corresp., 1948) before they become unduly elongated. One series of stipulodes (*haplostephanous,* Pl. I, Fig. 3-A) or two series (*diplostephanous,* Pl. I, Fig. 3-C) may develop from axial nodal peripheral cells and subtend the branchlets. Cortical nodal cells divide longitudinally into an inner and outer cell. The outer cell may merely swell into a *papillus* or *spine,* or may cut off several elongated processes (*spines*) in groups of 2–5 (*fascicled,* Pl. IV, Fig. 2-B). Gametangia borne primarily at the branchlet nodes; in monoecious species the antheridium is generally directly below the oogonium (*conjoined*), or isolated at a separate node (*sejoined*); in dioecious species borne on separate plants. Antheridia arise from peripheral nodal cells of branchlet nodes. Oogonia (in conjoined monoecious species) arise from laterals of antheridial stalk, on the abaxial side; spine-like processes (*bracteoles,* Pl. I, Fig. 2-C) which develop from cells of the antheridial lateral appear to subtend the oogonium. These may closely resemble the bracts. Starch bulbils regularly formed on rhizoids of certain species. Oospores terete, not laterally compressed.

5. **Chara Braunii** Gmelin, Fl. Badensis Alsatica 4: 646. 1826.
Chara coronata Ziz. (ined., *c. annum* 1814); Braun, Ann. Sci. Nat., ser. II, 1: 353. 1834.
References for the region: *C. coronata:* Owen (1888: 75); var. *Schweinitzii:* Halsted (1879: 181); Owen, *l.c.*

(Plate II, Fig. 7; Plate IV, Fig. 1)

Plant monoecious, totally ecorticate, 20 cm. high. *Branchlets* 8–9 in a whorl, widely spreading to closely convergent; articulations of branchlets (3–) 4 (–5), first and second nodes generally fertile; bracts 300–455 μ long to greatly reduced; terminal internodal cell occasionally somewhat inflated, terminated by a crown of 3 mucro-like cells, each 150–274 μ long. *Stipulodes* in one series (haplostephanous), 730–1035 μ long, alternating with the branchlets, spreading. *Gametangia* at first and second branchlet nodes, fundamentally conjoined, but antheridia frequently early deciduous; occasionally the sex organs geminate (2 oogonia and antheridia at a node). *Oogonia* 660–960 μ long by 384–490 μ broad; coronula about 150 μ long by 90 μ broad, the cells with short, divergent tips; convolutions 10; bracts about equal in length to the oogonia. *Oospores* 470–590 μ long by 259–340 μ broad, black; striae 8–10; membrane smooth and nearly opaque. *Antheridia* 268–282 μ in diameter. (Descr. from *Hannah Croasdale* (*2008* RDW); details of the gametangia from *Urda K. Wood,* July 16, 1947.)

PLATE IV



Variations among the local forms result from differences in development of vegetative structures. In size, some plants reach 35 cm. in height. The general plant structure varies from diffuse, attenuate forms, as in *Hannah Croasdale (2020* RDW), typical of relatively deep, quiet water to short, compact forms as in *R. D. Wood 2033*, characteristic of shallow littoral habitats The bracteoles vary from shorter than mature oogonia in *R. D. Wood 2005* to 1½ to 2 times as long in *Hannah Croasdale (2020* RDW). The crown of end cells terminal on branchlets varies in length from equal to the diameter of the branchlet in *R. D. Wood 2005* to less than half the diameter in *R. D. Wood 2029*. The relative lengths of the branchlet articulations vary from those in which the basal internode is about half the length of the others in *R. D. Wood 2005*, to those in which this articulation is extremely short in (*Coll.?*, Aug.), to those in which the terminal articulation is shortened and sometimes swollen in *B. F. D. Runk 457*. The elongation of the bracts at the primary branchlet nodes varies from about equal to other bracts in *R. D. Wood 2033* to those in which these bracts are twice the length of the bracts of other nodes in *B. F. D. Runk 457*. The stipulodes vary greatly in length on the same specimen, but are characteristically short in *Hannah Croasdale (2008* RDW) and long in *B. F. D. Runk 457*. The apparent general trend is for plants growing in shallow littoral zones to be tufted, form tightly connivent whorls, have shortened branchlet articulations and elongated bracts and stipulodes. Plants of deep, quiet water tend to be diffuse, have elongated branchlet articulations and reduced bracts, bracteoles, and stipulodes. These appear to be ecological variants, and range from what has been considered var. *Schweinitzii* Braun, in which bracteoles exceed mature oogonia in length, to var. *Braunii* (Braun) Zanev., in which bracteoles are equal to or shorter than mature oogonia. Oospores vary in length from 450–600 μ and thus are intermediate between the limits of var. *Schweinitzii* (550–650 μ) and var. *Braunii* (420–550 μ) as delimited by Zaneveld (1940: 139). It is thus concluded again (cf. Wood, 1947: 250; Allen, 1882b) that attempts to differentiate varieties of this species collected from various localities of North America are impossible in view of the facts. The writer has made a practice of annotating extreme specimens as approaching either the one variety or the other, but emphasizes that this is but a convenient manner of indicating differences in expression of the

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PLATE IV

All figures have been drawn with the aid of a camera lucida from specimens preserved in 2–3 per cent formalin. The indicated specimens are extant in the writer's herbarium. × 14.

FIGURE 1. *Chara Braunii* Gm. **1-A**, terminal cells of branchlet. **1-B**, axial node showing stipulodes at base of branchlets and lack of cortication. **1-C**, oogonium. **1-D**, oogonium subtended by antheridium. **1-E**, bracts at sterile branchlet node. (*Hannah Croasdale (2008* RDW).)

FIGURE 2. *Chara canescens* Lois. **2-A**, terminal cells of branchlet. **2-B**, axial node showing two rows of stipulodes, spines in fascicles, and haplostichous cortication. **2-C**, bracts of sterile branchlet node. **2-D**, oogonium at fertile branchlet node. **2-E**, axial cortication. (*2004*.)

FIGURE 3. *Chara delicatula* C. Agardh. **3-A**, terminal cells of branchlet. **3-B**, axial node showing one row of stipulodes and diplostichous tylacanthous cortication. **3-C**, fertile branchlet node with oogonium and antheridium. **3-D**, fertile branchlet node with an oogonium. **3-E**, axial cortication. (*2002*.)

FIGURE 4. *Chara delicatula* C. Agardh var. *barbata* Groves & B.-W., *prox.* **4-A**, terminal cells of branchlet showing elongated penultimate cell. **4-B**, axial node showing well-developed two rows of stipulodes, and nearly isodiametric primary and secondary cortical cells. **4-C**, bracts at sterile branchlet nodes. **4-D**, axial cortication. (*2024*.)

vegetative characteristics. One peculiar specimen was found (*Coll.?*, Aug.), which had a definite glomerate appearance. This resulted from the development of a somewhat reduced branch at each node, combined with elongation of bracts and stipulodes, and typical compactness of shallow-water forms. No further collections of this form have been seen; and, until other specimens have been reported, it will be considered an anomaly.

This species is the favorite local Charad for physiological investigation, and investigators commonly mistake the plants for a Nitella. There should be no confusion once it is recognized that this species is an ecorticate Chara, and that it is the only local species of Characeae which is ecorticate, has stipulodes at the base of the branchlets, and in which the branchlets are not divided. The latter two characters easily distinguish the species in the field.

Illustrations: Allen (1882b); Groves & Bullock-Webster (1924: pl. 26); Woods (1894: pl. 30); Migula (1897: 324; 1925 226, fig. 1, 2).

Exsiccatae: Phyc. Boreali-Amer., No. *822; 1383* as *C. Schweinitzii;* Amer. Alg., No. *256, 529;* Char. Amer. Exsicc., No. *8, 12, 13, 14*.

Localities: Cape Cod: Chara Haven—*R. D. Wood 2005,* July 7, 1947 (RDW, ETM); Chara Pond—*B. F. D. Runk 406,* June 20, 1941 (*1099774* F, RDW); Desmid Haven—*E. T. Moul,* July 11, 1945 (ETM); Harper Pond—*J. J. Copeland,* July, 1928 (*13800* WRT); Ice House Pond—*M. S. Doty,* July 10, 1946 (*6624* MSD); Little Pond—*R. D. Wood 2033,* Aug. 1, 1947 (RDW, NY); Nobska Pond—*W. G. Farlow,* Sept. 15, 1876 (FH); Oyster Pond—*Urda K. Wood,* July 16, 1947 (RDW, preserved); Salt Pond—*R. D. Wood 2034,* July 30, 1947 (RDW); "Sandwich Pond," pond on W. side of road, route No. 6, E. of Sandwich—*R. D. Wood* & *Urda K. Wood* (*2007* RDW); Weeks Pond—*Hannah Croasdale,* July 14, 1946 (*6613* MSD), July 28, 1947 (*2020* RDW); *R. D. Wood 2017,* July 26, 1947 (RDW); Cuttyhunk: Gosnold Pond—*W. R. Taylor,* July 6, 1921 (*3670* WRT); Sheep Pond—*I. F. Lewis* (MBL); *W. R. Taylor,* July 9, 1925 (*10173* WRT, MICH); *R. D. Wood 2027* (RDW) and *2029* (RDW, NY), July 31, 1947; Nantucket: Long Pond—*W. H. Sheldon,* Aug. 7, 1934 (MMA); Sesachacha Pond—[*coll.?*], Aug. (NY); Weweeder Pond—[*T. Morong*] *2a,* July 15, 1887 (NY); Naushon: French Watering Place—*W. R. Taylor,* July 12, 1921 (*3669* WRT); July 13, 1922 (*7585* WRT); Pasque: West End Pond—*B. F. D. Runk 457,* July 1, 1941 (*1099770* F, RDW); ? Pond, small pool—2nd or 3d pool south of West End Pond—*W. R. Taylor,* July 2, 1941 (FH, *20841* WRT); ? Pond, smaller pond near the southwest end of island—*Hannah Croasdale,* July 6, 1933 (*16982* WRT); Penikese: Typha Pond—*Hannah Croasdale,* July 6, 1947 (*2008* RDW, MBL, MSD).

6. **Chara canescens** Desvaux *et* Lois. *apud* Loiseleur-Deslongchamps,[4] Not. Fl. France, p. 139. 1910.

Chara crinita Wallr., Annus Bot., p. 190. 1815.

Chara crinita var. *americana* Allen, Char. Amer., Part 2, plate 2. 1879.

References for the region: *Chara crinita:* Owen (1888: 75); *C. leptosperma:* Allen (1880: 5; 1882a: 41); Owen, l.c.; *C. canescens:* Croasdale (1935: 95).

(Plate II, Fig. 6; Plate IV, Fig. 2)

Plant dioecious, 12 cm. high, densely hirsute. *Main axes* haplostichous, cortical nodes bearing spines solitary to fascicles of 2–5, one secondary cortical cell commonly apparent at cortical nodes and extending 1/8 to 1/4 the way to the next node (some-

[4] Loiseleur-Deslongchamps has consistently been given the credit for this species. In a footnote (p. 135), he stated that Desvaux had recognized several new species in the genus Chara and desired that they be published for him pending a more extensive comparative examination of the species. The present species, however, was entitled "Chara canescens. N."; and since "N." indicates *nobis* (by us), both men are necessarily recognized in the author citation.

times greatly developed so as to give a nearly diplostichous appearance). *Branchlets* 6–8 in a whorl, articulations 4–6, corticated except for terminal 1–2 cells, terminal cell rounded at tip; bracts varying widely in length, blunt *Stipulodes*, diplostephanous (in 2 series), 2 pairs at each branchlet, upper series 297–447 μ long, generally exceeding the lower series in length, blunt. *Gametangia* borne on separate plants, i.e., dioecious. *Oogonia* about 890 μ long by 442 μ broad, borne regularly at first branchlet node, occasionally at second and third; convolutions 10; coronula short and broad, truncate, 86 μ tall by 147 μ broad; bracteoles blunt to acute, about equal to oogonia in length. *Oospores* about 590 μ long by 318 μ broad, black; striae about 10; membrane opaque. *Antheridia*, none seen on local specimens. (Descr. from *R. D. Wood 2004* (RDW).)

Little variation is exhibited among the local specimens collected. In size, some plants reach 20 cm. in height. The spines on the axes vary from very abundant and nearly twice as long as the diameter of the axis in *T. F. Allen*, Aug. 30, 1895, to rather sparse and shorter than the diameter of the axis in *R. D. Wood 2004*. Bracteoles vary from about equal in length to the mature oogonia in *R. D. Wood 2004*, to one to two times the length in *F. S. Collins*, July 25, 1882. The differences in expression of length of stipulodes, bracts, and spines appear to be more directly correlated with local physiochemical factors, particularly salinity, rather than depth as in *C. Braunii*. This follows logically since most local specimens of *C. canescens* have been found growing in water less than one foot deep. All the local specimens seen exhibited the angular oospores which characterize the form *leptosperma* Braun (1834: 356) as described by Allen (1882a: 41, pl. XVIII), but the present writer prefers to temporarily ignore forms as nomenclatural entities.

This species is highly characteristic in its very spinous appearance of the main axes. It is restricted to brackish-water ponds. Salinity determinations by means of silver nitrate titrations on fresh samples of water from various habitats during July and August, 1947, showed extremes of 4 to 22 o/oo NaCl, with the optimum vegetative development at about 10 o/oo NaCl.

Although no antheridia were discovered on local specimens, a very similar monoecious species, apparently *C. evoluta* Allen, was found in 1948 by Dr. V. I. Cheadle and Dr. E. A. Palmatier in Little Compton, Rhode Island, just across the state line of Massachusetts.

Illustrations: Allen (1879: pl. 2; 1880: pl. 2); Migula (1897: 352, 353; 1925: 226, fig. 6); Groves & Bullock-Webster (1924: pl. 27).

Exsiccatae: Phyc. Boreali-Amer., No. *823* as *C. crinita*.

Localities: CAPE COD: Chara Pond—*J. M. Fogg, Jr.*, June 29, 1925 (MBL, *10240* WRT); *W. R. Taylor*, July 30, 1937 (*17937* WRT, NY, FH); *H. T. Croasdale*, July 14, 1946 (*6612* MSD); *R. D. Wood 2004*, June 28, 1947 (RDW, MBL, MSD, ETM); Little Pond—*F. S. Collins*, July 25, 1882 (*19776* WRT, RDW, NY, *132461* UC; dupl. herb. T. F. Allen, as *C. crinita*); *W. A. Setchell* and *W. J. V. Osterhout*, Aug. 17, 1895 (MBL); *F. S. Collins*, July, 1886 (Y); July 25, 1882 (NY, *132461* UC, RDW); Salt Pond—*Urda K. Wood*, June 28, 1947 (*2003*, RDW); *R. D. Wood 2023*, July 30, 1947 (RDW, NY); ? Pond, Woods Hole—*J. J. Copeland* (*16772* WRT); NANTUCKET: Coskata Pond—*T. Morong 5*, July 15, 1887 (NY); *W. H. Sheldon*, Aug. 6, 1931 (MMA); [*coll.?*], Aug. 21, 1896 (MMA); Polpis—*Maria Owen*, July, 1879 (NY); Sesachacha Pond—*Dame, Jenks* and *Swan*, July 14, 1880 (NY); *F. S. Collins*, July, 1886 (Y, NY, *7929* WRT, *823* P. B.-A. as *C. crinita* f. *leptosperma*); *L. L. Dame*, Aug. 1886 (NY); *E. P. Bicknell 11633*, Sept. 16, 1899 (NY); Wawinet—*T. F. Allen*, Aug. 30, 1895 (*132435* UC, NY, *19782* WRT, RDW; dupl. herb. T. F. Allen, as *C. crinita* Wallr., det. T. F. Allen); ? Pond—*L. L. Dame*, Aug., 1880 (NY); July 11, 1886 (NY).

7. **Chara delicatula** C. A. Agardh, Syst. Alg., p. 130. 1824. (*nom. illeg.*) [5]

(*Non*) *Chara delicatula* Desvaux *ex* Lois., Not Fl. France, p. 137. 1810 (= *C. aspera* (Dethard.) Willdenow, *fide* Migula (1897: 654)).

Chara fragilis var. *delicatula* von Leonhardi, Verhandl. Naturf. Vereins Brunn 2: 209 1864

Chara verrucosa Itzigsohn, Bot. Zeit. 8: 338. 1850, *fide* Robinson (1906: 280).

References for the region: *Chara fragilis:* Croasdale (1935: 96 [pl. not seen]); var. *delicatula:* Owen (1888: 75); *C. delicatula:* Croasdale (1935: 96).

(Plate II, Fig. 5; Plate IV, Fig. 3, 4)

Plant monoecious, 10 cm. high; slightly incrusted with lime; starch bulbils abundant on rhizoids. *Main axes* regularly triplostichous, secondary cortical cells generally somewhat smaller in diameter than the primary cells; spines at cortical nodes reduced to mere papillae. *Branchlets* 8 in a whorl, articulations 7–10, corticated except for terminal 1–2 cells; bracts at sterile nodes inconspicuous. *Stipulodes* apparently haplostephanous, reduced to mere papillae at some nodes *Gametangia* conjoined at branchlet nodes, but frequently one or other sex appears to be lacking. *Oogonia* 850–1000 μ long by 450–500 μ broad; convolutions 12, very nearly perpendicular to long axis of oogonium; coronula elongate at maturity, 250–270 μ tall, cells generally connivent. *Oospores* 460–500 μ in diameter, black; striae 12–13; membrane opaque. *Antheridia* 410–455 μ in diameter. (Descr. from *R. D. Wood 2032* (RDW).)

Variations among the local forms are numerous, and result from differences in expression of the vegetative characteristics. In size, some plants reach 25 cm. in height. Other variations appear to be of two distinct orders: (1) a remarkably constant differentiation into forms with two well-developed series of stipulodes (diplostephanous) as in *R. D. Wood & Urda K. Wood (2058* RDW) and forms with one well-developed series (haplostephanous) as in *R. D. Wood 2032;* and (2) less constant differences which appear in random combination. The haplostephanous form is the typical form as described by Groves and Bullock-Webster (1924: 65); whereas the diplostephanous form (Pl. IV, Fig. 4) agrees closely with the description of var. *barbata* (Ganterer) Groves and Bullock-Webster (1924: 68, pl. 44, fig. 9). The features subject to less constant variation are several. In general habit, the plants vary from very attenuate form in *W. R. Taylor* (*10171* WRT) to densely compact form in *R. D. Wood 2000.* The number of ecorticated terminal branchlet articulations varies from 3–6 in *R. D. Wood 2002* to 1–2 in *W. R. Taylor* (*10171* WRT). Generally, the penultimate cell of branchlet is not swollen, but it may be rather inflated as in *R. D. Wood 2022* or very elongate as in *R. D. Wood 2002.* The general trend in expression, as in *C. Braunii,* is toward more attenuate

[5] The problem of just what is the valid name for this species is one of long standing. The plant has become known as *C. delicatula* C. Ag., but this name is a later homonym of *C. delicatula* Desv. (= *C. aspera, fide* Braun; cf. Groves & Bullock-Webster, 1924: 51, 67). Robinson (1906: 280) decided upon *C. verrucosa* Itzigsohn (1850: 338), but it is not known if he saw the type specimen of that plant. There are at least three synonyms in the literature prior to 1850, including *C. pilifera* C. Ag. (1824: xxviii), *C. virgata* Kutzing (1834: 56), and *C. foliolata* Hartman (1820: 378). The problem can be solved only by inspection of the type specimens, a task most easily accomplished by students in the countries in which the critical specimens are to be found.

plants with longer branchlet articulations, shorter bracts, bracteoles, and stipulodes in quiet, deeper water; and compact, tufted, frequently bulbiliferous (with abundant starch bulbils on rhizoids) plants with longer bracts, bracteoles, and stipulodes in shallow littoral.

This species varies widely, and approaches *C. globularis* Thuill. (= *C. fragilis* Desv.) very closely. At one extreme, it is almost identical in vegetative characters to *C. aspera*. Croasdale's (1935) record of *C. fragilis* for Falmouth Heights was probably *C. delicatula*.

Illustrations: Typical form—Groves, H. & J. (1880: pl 207, fig. 1a); Migula (1897: 753; 1925: 242, fig. 7); Groves & Bullock-Webster (1924: pl. 44, figs. 1-8). Var. *barbata*—Groves & Bullock-Webster (1924: pl. 44, fig. 9).

Exsiccatae: Phyc. Boreali-Amer., No. *1199* as *C. fragilis* subsp. *delicatula;* Char. Amer. Exsicc., No. *22*.

Localities: Cape Cod: Chara Haven—*R. D. Wood 2010,* July 7, 1947 (RDW, NY); *R. D. Wood* & *Urda K. Wood*, Sept. 6, 1947 (*2058* RDW); Chara Pond—*M. S. Doty,* June 28, 1947 (*2001* RDW, MBL, MSD, ETM); *R. D. Wood 2002,* June 28, 1947 (RDW, NY); Little Pond, Falmouth Heights—*R. D. Wood 2006,* July 7, 1947 (RDW, NY), *2032,* Aug. 1, 1947 (RDW); Oyster Pond Annex [?], S.E. of Oyster Pond—*W. R. Taylor,* July 4, 1925 (*10172* WRT, MICH); Salt Pond—*W. R. Taylor,* July 15, 1917 (*2294* WRT); *Hannah Croasdale,* July 14, 1946 (*6614* MSD); *R. D. Wood 2000,* June 28, 1947 (RDW, NY), *2018,* July 26, 1947 (RDW), *2024, 2025* (NY, RDW), *2022, 2026,* July 30, 1947 (RDW); Cuttyhunk: Gosnold Pond—*W. R. Taylor,* July 15, 1919 (*2931* WRT); Sheep Pond—*W. R. Taylor,* July 9, 1925 (*10171* WRT), June 28, 1932 (*17012, 17083* WRT, NY), July 3, 1934 (*16767, 16766* WRT); *R. D. Wood 2028,* July 31, 1947 (RDW, NY), collected on inland edge of pond, *2029,* July 31, 1947 (RDW, NY) collected on seaward edge of pond; Nantucket: Sesachacha Pond—*L. L. Dame,* July, 1886 (NY); *F. S. Collins,* July, 1886 (NY); Naushon: French Watering Place—*W. R. Taylor,* July 6, 1920 (*3105* WRT).

8. Chara aspera Willd., Ges. Naturf. Fr. Berlin 3: 298. 1809.

The writer has seen no herbarium specimens which are undisputably of this species for the region, and as such regards the local records as highly questionable. Of the specimens seen, the sterile condition rendered differentiation from *C. delicatula* almost impossible, and recently collected fertile specimens have all proved to be *C. delicatula* Ag. However, during the summer of 1948, Dr. E. A. Palmatier and Dr. V. I. Cheadle collected excellent male and female specimens of *C. aspera* in Rhode Island just across the Massachusetts state line in Ashawonk's Swamp, Little Compton. The chances are good that *C. aspera* should be found in our region in brackish-water ponds.

The best practical distinguishing feature between the American forms of these two species seems to be the fact that *C. aspera* is dioecious, whereas *C. delicatula* is monoecious. A generalized description of *C. aspera* is almost identical to *C. delicatula* in vegetative parts. In *C. aspera*, the female plant has whorls of branchlets which are more spreading than the closely convergent branchlets of the male plants. Antheridia are borne at most of the nodes of fertile branchlets of male plants. Oogonia are borne at the first three (occasionally fourth) fertile nodes of female plants. G. O. Allen (corresp., 1948) stated that normally *C. aspera* has long spine-cells [spines] which are short in *C. delicatula.* The writer has studied the New England specimens, particularly those for Massachusetts and Rhode Island, and concludes that this is helpful but finds that in this region *C. aspera* may be practically devoid of spines; hence, this character of European plants does not appear to hold for our specimens.

Illustrations: Allen (1882b: pl. 21, fig. A, 1–3, 7), Migula (1897: 656, 657); Groves & Bullock-Webster (1924: pl. 39)

Exsiccatae: Phyc. Boreali-Amer., No. *1196;* Char. Amer. Exsicc., No. *26, 27.*

Localities: none known.

Species Excluded from Local Flora

Chara fragilis Desv. (= *C. globularis* Thuill.); from Chara Pond, Cape Cod, *fide* Croasdale (1935) = ? *Chara delicatula* Ag. (specimen not seen by author).

Nitella batrachosperma (Reich.) Braun (= *N. Nordstedtiana* Groves, H. & J.); from Nantucket, *fide* Owen (1888, determined by T. F. Allen) = *N. Morongii* Allen *emend.* Wood.

Nitella gracilis (Sm.) C. Ag.; from Wood Pond, Golf Pond, Cape Cod, *fide* Croasdale (1935), Nobska Pond, *fide* F. S. Collins (P. B.-A., No. *1195*) = *N. Morongii* Allen *emend.* Wood.

Nitella maxceana Allen, *spec. dub.;* from [Maxey's Pond] Nantucket, *fide* T. F. Allen (1896) = *N. Morongii* Allen *emend.* Wood.

Nitella mucronata (Braun) Miquel var. *gracillima* Groves & Bullock-Webster; from Wood Pond, Cape Cod, *fide* Croasdale (1935) = *N. megacarpa* Allen.

Table I

	Nitella flexilis	Nitella megacarpa	Nitella Morongii	Nitella translis	Chara Braunii	Chara canescens	Chara delicatula
Cape Cod							
Ashumet Pond	0	P	0	X	0	0	0
Chara Haven	0	0	0	0	X	0	X
Chara Pond	0	0	0	0	X	PX	X
Desmid Haven	0	0	0	0	X	0	0
Golf Pond	0	0	P	0	0	0	0
Harper Pond	0	0	X	0	P	0	0
Ice House Pond	0	0	0	0	X	0	0
John Pond	0	0	0	P	0	0	0
Leech Pond	0	P	0	0	0	0	0
Little Pond	0	0	0	0	X	P	X
Nobska Pond	0	0	P	0	P	0	0
Oyster Pond	0	0	0	0	X	0	0
Oyster Pond Annex	0	0	0	0	0	0	P
Salt Pond	0	0	0	0	X	X	PX
Sandwich Pond	0	0	0	0	X	0	0
Summerfield, South	0	P	0	0	0	0	0
Weeks Pond	X	X	0	X	X	0	0
Wood Pond (Gansett Pond)	0	P	0	0	0	0	0
Cuttyhunk							
Clubhouse Pond	X	0	0	0	0	0	0
Gosnold Pond	0	0	0	0	P	0	P
Sheep Pond	P	0	0	0	PX	0	PX
Sheep Spring	P	0	0	0	0	0	0
Martha's Vineyard							
Chilmark Pond	X	0	0	0	0	0	0
Tiasquam Dam	X	0	0	0	0	0	0

TABLE I—*Continued*

	Nitella flexilis	Nitella mega-carpa	Nitella Morongii	Nitella transilis	Chara Braunii	Chara canescens	Chara deli-catula
Nantucket							
Cato's Pond	P	0	0	0	0	0	0
Coskata Pond	0	0	0	0	0	P	0
Long Pond	0	0	0	0	P	0	0
New Lane Pond	X	0	0	0	0	0	0
Maxey's Pond	0	0	P	0	0	0	0
Polpis	P	0	0	0	0	P	0
R. R. Track Pond	P	0	0	0	0	0	0
Sesachacha Ditch	X	0	0	0	0	0	0
Sesachacha Pond	0	0	0	0	P	P	P
Siasconset Pond	0	0	P	0	0	0	0
Wawinet Pond	0	0	0	0	0	P	0
Weweeder Pond	P	0	0	0	P	0	0
Naushon							
French Watering Place	0	0	0	0	P	0	P
Petchett (Peckett) Pond	0	0	X	0	0	0	0
Nonamesset							
South Pond	P	0	0	0	0	0	0
Pasque							
West End Pond (Nitella Pond)	X	0	0	0	X	0	0
? Pond, near West End Pond	0	0	0	0	X	0	0
? Pond, smaller pond near S. W. end of island	0	0	0	0	P	0	0
? Pond, near west end of island	X	0	0	0	0	0	0
Penikese							
Typha Pond	0	0	0	0	X	0	0

LOCALITIES FOR CHARACEAE IN WOODS HOLE REGION

In the above table, all localities are listed from which specimens of Characeae have been seen by the writer. The substantiating specimens are listed in the text under "localities" for each species. Because of the great effect of the hurricane of 1938 on the coastal ponds of the region, the writer has chosen this date as the critical year. Specimens collected prior to 1938 are recorded as "P." Specimens collected since 1938 are recorded as "X." If no collections have been seen by the writer, this is recorded as "0."

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RADIOACTIVE SODIUM PERMEABILITY AND EXCHANGE IN FROG EGGS

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Statement of the Problem

The transfer of ions in both directions across the cell membrane is a matter of major biological importance. While problems involving permeability have led to a vast and prolix literature, only meager attention has centered on the specific distribution of electrolytes within single cells. The limitations of earlier techniques were emphasized by Hastings (1941) in his Harvey lecture on the tissues and body fluids, in which he early recognized the value of radioactive isotopes. The subject of permeability has been recently reviewed by S. C. Brooks (1945). Our interest has centered in the dynamic equilibria of a protoplasmic system, their control by diffusion and the blocking of internal exchange. Use of radioactive tracer techniques has given new precision to the qualitative and quantitative study of small ionic transfers within cellular dimensions. Accordingly, in this paper are given results of an investigation undertaken to determine how much and how fast traced sodium (containing Na^{24}) exchanges with normal sodium (Na^{23}) in a vertebrate egg.

Materials and Methods

Ovarian "winter" eggs of the frog, *Rana pipiens,* were chosen because they are single cells, spherical and of large size. Throughout the fall and winter quarters of the year, they are readily available in quantity (without hormone stimulation). In addition to the vitelline membrane, ovarian eggs possess two thin epithelial layers which could be removed successively by fine-pointed forceps. However, in most of the experiments, these membranes were left intact, because of the possibility of injury to the cell surface. Amphibian eggs are ideal for volume measurements. One unique advantage is that they possess an extremely large nucleus, or germinal vesicle, approximately 0.5 mm. in diameter, which may be isolated easily under a dissecting microscope in a Ca-free medium (Duryee, 1937).

Eggs were isolated individually in standard Ringer solution: NaCl, 0.66 g.; KCl, 0.014 g.; $CaCl_2$, 0.012 g.; distilled H_2O, 100 ml. All solutions were buffered to pH 7.6 with $NaHCO_3$. As described below, Na^{24} was incorporated in experimental solutions as NaCl.

Active healthy frogs were selected. After ovaries of pithed animals were removed as bloodlessly as possible, fresh Ringer solution was poured over them before transfer to small beakers. A small lobe of an ovary was next cut off with iridectomy scissors taking care not to injure the cells. Groups of 20–30 eggs were next transferred to Syracuse dishes where individual eggs were cut apart. All small yellow or transparent eggs were removed and the ovarian wall was trimmed off flush with the theca membranes of each full-size egg. In this way, using a dissecting microscope to insure precise excision, it was possible to obtain from 40 to 60 separate eggs per hour. Average volume was 2.86 mm^3, based on an average diameter of 1.76 mm. An important step was the reexamination of individual eggs to reject those which might have been nicked or crushed. Aspherical eggs were also discarded along with any less than 1.65 mm. in diameter. Following these procedures, a final screening for uniformity of diameter was made. This consisted of shaking approximately 100 eggs in a flat bottom shallow dish to obtain a single layer with close packing. Any cell not obviously tangent to its six neighbors was discarded.

Eggs were conveniently handled individually with a pipette having an internal diameter just larger than that of the cell itself. In a few experiments where short exposures involving minimum transfer of external radioactive material was essential, each egg was advantageously picked up by the short flap of theca membrane with sharp forceps.

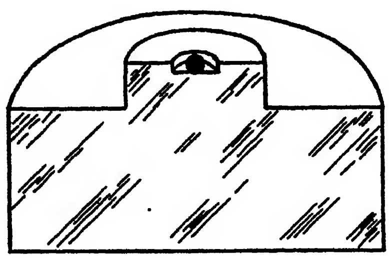

FIGURE 1. Sectional view of plexiglas cup for measuring radioactivity in frog egg. Actual size.

For experiments on dead cells, a method of killing involving minimal change was sought. After rejecting reagents such as alcohol, acetic acid and KCN, we standardized on heating to 65° C. for ten minutes in normal Ringer.

Groups of 10–20 eggs were exposed in small beakers containing 20 ml. of Ringer. Temperature was maintained in water baths controlled to ± 0.5° C. The presence of water vapor surrounding the beakers kept down evaporation, which by weighing was found to be negligible over periods up to 24 hours.

Upon removing a cell from the radioactive Ringer, it was put through two washes of normal Ringer before transfer to the measuring cup. One or two eggs were first transferred in a vertically held pipette allowing them to sink to the meniscus. By merely touching the surface of the washing fluid, the eggs dropped

from the pipette, thus avoiding transfer of more than one or two cubic millimeters of previous fluid. Washing vials each held 7 ml. It was considered advisable to use a fresh pipette for each successive transfer. Since it was found that Na^{24} could be washed from the cell interiors, the process was reduced to a standard thirty seconds.

For measurement in the Geiger counter, eggs were transferred from the second washing vial to special plexiglas cups (see Fig. 1) again in a vertically held pipette. Not more than 5 mm^3 of the second wash fluid was thus transferred with the egg. Tests of this fluid showed negligible radioactivity.

Radioactive solutions

The radioactive Na^{24} was prepared in the cyclotron by bombardment of metallic sodium with 16 m.e.v. deuterons. The metal was dissolved in ethyl alcohol and converted into NaCl with 12 N hydrochloric acid. The mixture was then dried and ignited at red heat to produce a neutral salt free of organic material. Weighed amounts of the radioactive salt were converted into amphibian Ringer solution. To obtain a relation between quantity and radioactivity of Na^{24}, a known aliquot of the Ringer was evaporated to dryness and measured with a Geiger counter. A typical result was as follows: 1 ml. of amphibian Ringer was diluted to 500 ml. A volume of 0.039 ml. of this solution was dried and measured on the counter. Since 1 ml. of the Ringer contained 0.026 g. Na, the aliquot possessed $0.026 \times 1/500 \times 0.039 = 2.03 \times 10^{-7}$ g. Na. This sample gave 1600 counts/min. Thus 1000 counts/min. represented 1.27×10^{-7} g. Na.

For each solution made up with radioactive sodium, controls were made up with identical amounts of normal NaCl. The radioactivity of the solutions was such that the radiation level was less than an amount which would produce 1 r.e.p./min. No visible cytological changes were produced by this level of radiation. In view of the well-known tolerance of frog eggs to much larger dosage of X-rays, it is fair to assume that radiological effects were negligible in our results.

Radio-autographs

A new variant of conventional methods of making radio-autographs has been devised to fit the purpose of recording radioactivity distribution within a single cell. Our technique consisted essentially of quick-freezing in liquid air, followed by sectioning the cell to a known thickness, and then exposing the section in a light-proof cold box to a photographic plate.

Eggs were rapidly rinsed (5 sec.) in two washes of normal Ringer (7 ml. each), oriented in a 1.8 mm. hole in thin calibrated bronze strips (1.5×3.5 cm. and either 80 or 200 μ thick) and plunged into liquid air. The orientation was such that the axis of the egg was parallel to the surface of the strip. It was calculated that freezing of a 1.7 mm. egg at $-180°$ C. would be completed at the center in about 0.2 sec. This time should be compared with the ten minutes necessary for partial equilibrium of ionic diffusion in the egg at normal temperatures. The bronze strip containing the frozen cell was then placed on a previously cooled brass block (see Fig. 2) and both were transferred to the stage of a dissecting microscope. Using a cooled razor blade mounted in a special non-conducting handle, the frozen egg was sliced down to expose the nucleus. Orientation was

thus re-checked to insure that the plane of section was being made parallel to the egg axis. Improperly oriented eggs were discarded. Subsequent slices through the lateral third of the nucleus were made, making the surface even and parallel with the bronze strip. The nucleus always stood out sharply as a white circle in a grey background of frozen cytoplasm. Finally the strip was inverted and the cell was sectioned similarly from the other side. Frozen sections were conveniently temporarily stored in a cold slotted brass holder in an insulated box, cooled with solid CO_2.

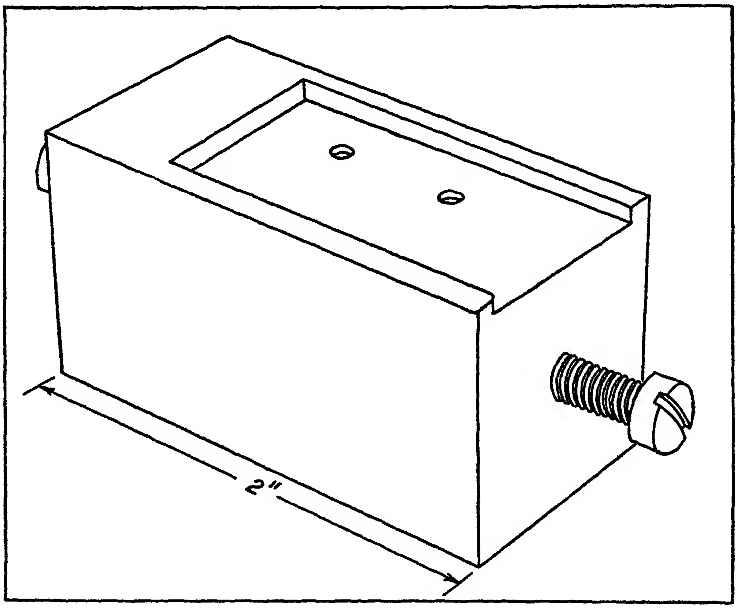

FIGURE 2. Brass cooling block for holding frozen egg and supporting strip during sectioning. Note that both holes corresponding to eggs in holder are displaced laterally from the main axis of the block, so that the strip, when inverted after first sectioning, brings cut surface against flat portion of the block. Screw at each end makes convenient attachment for handle.

Actual exposure of the section to film took place in a well-insulated chest fitted with a copper bottom and copper slide holder. One hundred pounds of dry ice kept the temperature in the slide holder at approximately $-40°$ C. for a week. Medium-contrast lantern slide plates [1] were laid in the slide holder, emulsion side up, and the cold bronze strips with the frozen sections were laid on the plates, being weighted to insure even contact. With the activity of our solutions, it was found necessary to expose from two to twelve hours to provide the desired darkening. Plates were developed in Eastman D-19 developer.

A typical calculation indicates the amounts of radioactivity involved. A section of a frog egg 80 μ thick contained 2.0×10^{-8} g. of exchanged sodium. This sample

[1] We have learned from Dr. Kenneth Endicott, of the National Institutes of Health, that Ansco non-screen X-ray films are particularly useful where only low intensities of radiation are present.

gave 320 counts/min. on the counter. The actual number of disintegrations occurring in the egg section was approximately 20/sec. An exposure of four hours sufficed to give a good darkening showing a clear differential distribution between nucleus and cytoplasm. This amounts to 288,000 electrons, about half of which went into 0.026 cm^2 of the plate emulsion.

Results

Sodium content of ovarian eggs

The sodium content of frog eggs is not well known. Bialaszewicz (1929) has given an approximate figure of 42 mg. Na/100 g. wet weight of eggs of *Rana temporaria.* It seemed desirable, therefore, to determine sodium on our experimental animals. For this purpose, both ovaries were removed from six animals. The ovaries were rinsed quickly in distilled water, blotted, weighed, and cautiously ashed over low heat by concentrated HNO_3, followed by concentrated H_2SO_4.

After removal of a small amount of insoluble material, probably $CaSO_4$, the sodium was determined as $(UO_2)_3ZnNa(CH_3COO)_9 \cdot 6H_2O$ according to the method of Barber and Kolthoff (as given in Hillebrand and Lundell, 1929). Results obtained from six pairs of ovaries gave a preliminary value of 137 mg. Na per 100 g. wet weight of eggs (Duryee and Abelson, 1947). Further study has shown these results to be too high and that PO_4 interferes with the accuracy of the method. This is at least in part due to the formation of an insoluble uranium-phosphorus compound which tends toward giving high results for the sodium value. Accordingly the procedure was modified. Following wet ashing, excess acid was driven off, the residue dissolved in distilled water, and the solution made alkaline with NH_4OH. A 25 per cent barium acetate solution was added until no further precipitate formed, followed by a few drops excess. The mixture was allowed to stand for half an hour and then centrifuged. The precipitate was washed twice with distilled water and the washings added to the first supernatant. The solution was evaporated to dryness, the residue dissolved in 1 ml. of distilled water, and the sodium precipitated with the uranyl zinc acetate reagent. The following results were obtained from six pairs of ovaries:

Wt. of ovaries (grams)	mg. Na	mg. Na/g. ovary
8.1	6.8	0.84
9.3	7.40	0.80
9.0	7.4	0.82
8.5	7.0	0.82
7.0	5.9	0.80
8.4	7.1	0.84
	Average	0.82 mg. Na

There remained the question of how well the sodium content of the whole ovary approximates that of the eggs. Since the connective tissue, blood vessels and peritoneum formed only a minor fraction of the ovary, it seemed that the error introduced by neglecting this factor would be small. A sodium determination on separated eggs gave 0.80 mg. Na/g. wet weight. Eggs averaged 1.76 mm. in diameter with a volume of 2.86 mm^3, a density of 1.10, and a calculated sodium content of 25.8×10^{-7} g. Na per egg.

Water content of eggs

In analyzing the results, it was found desirable to measure the water content of the eggs. Accordingly four determinations were made—two with separated eggs and two with ovaries. Excess moisture was blotted up with filter paper. The eggs were weighed and then dried to constant weight at 120° C. for one hour. A water content of 52 per cent was found. This corresponded to 1.63 mg. of water for our standard 1.76 mm. diameter egg.

An interesting observation gave evidence on the water content of the nucleus. In six sets of eggs which were frozen whole, the half sections, when thawed and dried, showed a negative mold of the nucleus with only a flake remaining. This indicates the relatively higher water content of the nucleus above that of the yolk-cytoplasm.

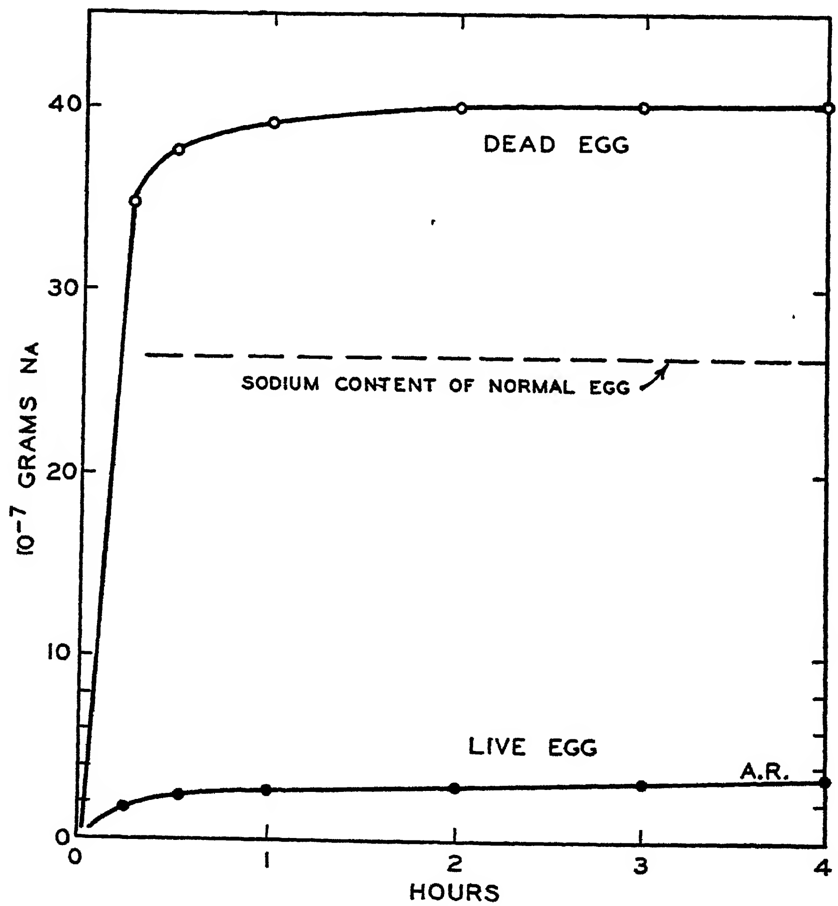

FIGURE 3. Comparison of uptake of Na^{24} by live and dead eggs in amphibian Ringer. Average value of sodium content of single egg, as determined by chemical analysis, shown by dotted line. Each point represents average measurements on ten separate cells.

Sodium exchange

In the course of the research a total of twenty experiments were performed involving 400 individual eggs. The experiments gave a reproducible set of values with a probable variation of plus or minus ten per cent. A comparison of the exchange of sodium in live and dead eggs is shown in Figure 3. Each point on the curve represents an average of ten measurements. In the case of live eggs, the following features are to be noted: Initially there was a rapid penetration of the traced sodium into the egg. Within an hour, however, the amount of the traced substance within the cell ceased to increase. The level reached amounts to 12 per cent of the total sodium content of the egg. By testing samples of cytoplasm and later by making radio-autographs, it was shown that the traced sodium was principally inside the egg.

With dead eggs (Fig. 3) the exchange of sodium was extremely rapid. The amount of traced sodium which could be found in the egg rose above the total sodium content of the live cell as determined chemically. This entrance of extra sodium can be related to at least two facts: We have observed a loss of potassium from the cell on death. The second fact is that the sodium content of amphibian Ringer for a volume equal to that of the frog egg is 78.4×10^{-7} g., or more significantly, the sodium present in a volume of Ringer equal to that of the cell's water content is 42.4×10^{-7} g. Na.

To study further the nature of the sodium uptake process, eggs were first exposed to radioactive Ringer, washed and then immersed in an inactive Ringer solution. The results are shown in Figure 4. During the first hour, a typical curve was obtained for penetration of the traced sodium. The downward curve was obtained during exposure to inactive Ringer. The time required for the traced sodium to leave the cell was about the same as the time involved in the initial penetration. Chemical analysis showed that the total sodium content of the egg did not change during these experiments. When dead eggs containing tracer sodium are exposed to inactive Ringer, the traced sodium likewise leaves the egg very quickly.

Since calcium is thought to be important in permeability relationships, experiments were designed to investigate the effect of this ion on sodium exchange. Three solutions were made up with tracer sodium: The first, an ordinary Ringer solution; the second, with calcium not present; and a third, with double the usual calcium content. When eggs were exposed to these solutions and measured as before, the curves shown in Figure 5 were obtained. These curves show that calcium only slightly influenced that fraction of the sodium (12 per cent) which is exchangeable. After several hours, however, lack of calcium resulted in an increased exchange of the sodium.

A series of experiments was made to determine the effect of temperature on exchange of sodium. Experiments were conducted at 9° C., 20° C., and 30° C. While the speed of penetration of sodium was somewhat faster at the higher temperature, the level reached after an hour was almost identical in all cases. After three hours approximately 6×10^{-7} g. Na was found per cell, while the low temperature values were essentially those of the controls at 20° C. It is apparent, therefore, that sodium exchange is not highly dependent on cellular metabolism.

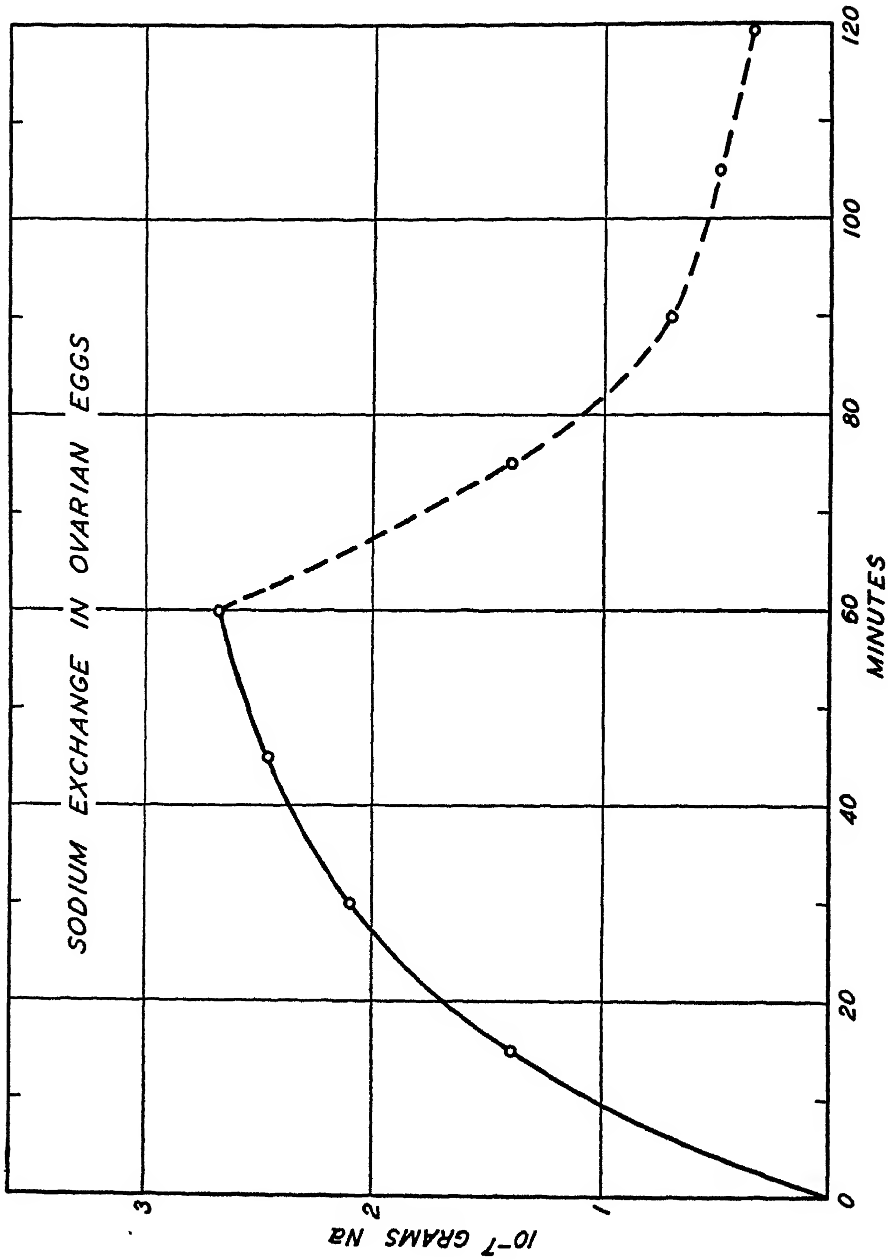

FIGURE 4. Comparison of rate of uptake of Na^{24} in a radioactive Ringer solution with the rate of "washing-out" in a *normal* Ringer medium.

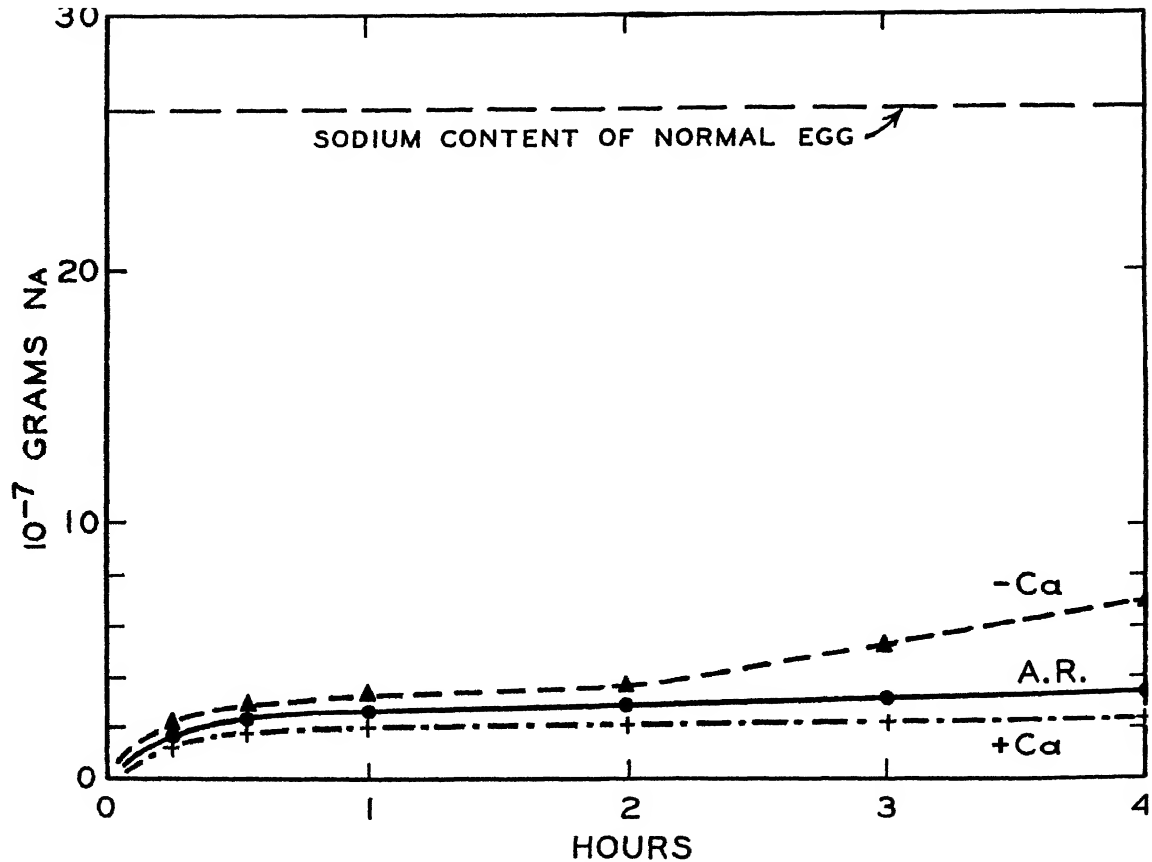

FIGURE 5. The influence of calcium on the rates of sodium exchange in single cells. Calcium-free Ringer (−Ca) ---; normal amphibian Ringer (Ar) solid line; Ringer with double calcium content (+Ca) -·-·-.

FIGURE 6 Radio-autograph of ovarian egg exposed to Na^{24} for thirty minutes Cell was frozen in liquid air and sectioned parallel to main axis Dark portion in upper third corresponds to size and position of the cell nucleus.

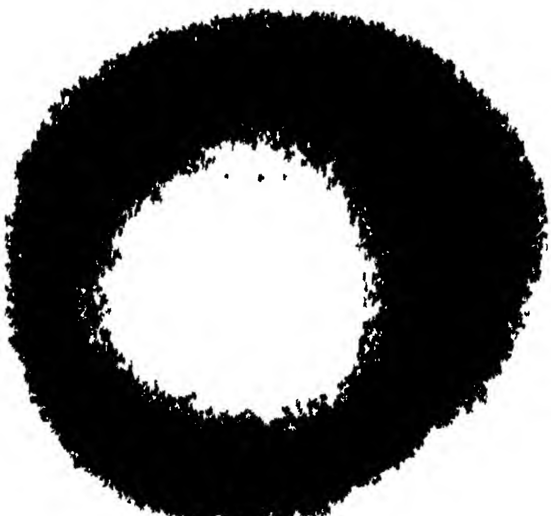

FIGURE 7. Radio-autograph of dead ovarian egg exposed to Na^{24} for five minutes. Width of "ring" is proportional to diffusion rate of sodium inside the cell.

The question of where the sodium goes inside a cell seemed of fundamental importance. Many attempts were made to answer this question with but little success. It was of course possible to isolate single nuclei from exposed eggs. Such nuclei were washed twice in calcium-free nuclear medium to remove outside "cytoplasmic" sodium and were then placed individually on plastic cups for measurement with the Geiger counter. The counts per nucleus were always significantly higher than background, but showed disappointing variation. It was concluded that the washing process introduced an uncontrollable variable. Consequently, we turned to the radio-autograph technique. In Figure 6 is shown an enlargement of a typical radio-autograph of a frog egg frozen in liquid air and sectioned meridianally. Of 47 autographs of single cells exposed to Na^{24} for more than 30 minutes, all showed denser silver particles in the nuclear area than in the cytoplasm, when the plane of section was through the germinal vesicle. In those autographs of eggs sectioned to one side of the nucleus, a uniform density was found throughout the endoplasmic area.

In the analysis of sodium diffusion rates inside the cell, advantage was taken of the radio-autographic records of penetration. In Figure 7 is shown an enlargement of a radio-autograph obtained by exposure of a dead egg to radioactive Ringer for five minutes followed by washing, freezing, and sectioning. When live eggs were exposed to the active Ringer, for five minutes, followed by standard technique, a similar ring-shaped autograph was also obtained with the density of the darkening much less.

Discussion

To serve as a basis of comparison, a calculation [2] has been made of the time required for diffusion processes to exchange sodium in an element of fluid the size

[2] An exact calculation of diffusion in an object similar to the frog egg would be practically impossible. First, there is the inhomogeneity of nucleus and cytoplasm. Second, is the fact that only a part of the sodium in the egg exchanges One important factor in the calculation is that these experiments trace the behavior of Na^{24} in a medium under conditions where there is no net transport of sodium or chloride ions. Therefore, in calculating the diffusion of Na^{24} in Ringer solution, one should use the self-diffusion coefficient of Na^+ rather than that of NaCl. Fortunately, the self-diffusion of Na has been measured in sodium chloride solutions by Jehle (1938) and in sodium iodide solutions by Adamson (1947). In the experiments the concentration of salt is the same on both sides of a diaphragm, and a very small amount of radioactive sodium is added to one of the solutions. From the rate of appearance of tracer sodium in the second solution the self-diffusion coefficient can be measured. The value observed was 1.2×10^{-5} cm^2./sec. in 0.113 M NaCl solution, and 1.23×10^{-5} cm^2./sec. in 0.113 M sodium iodide solutions.

A calculation giving useful information regarding diffusion into a sphere can be made by employing formulas presented by Barrer (1941). The formulas have been applied to "Ionic Exchange Absorption Processes" by G. E. Boyd et al. (1947). In the case of a completely permeable sphere initially free of the diffusion ion, the time required for the solute to reach half of its equilibrium value is given by

$$Q/Q_\infty = 1/2 = 1 - 6/\pi^2 \sum_{n=1}^{\infty} 1/n^2 \exp.\ (-D\pi^2 n^2 t/r^2)$$

where Q_∞ is the equilibrium value of the quantity of traced sodium, Q is the value at any time t, D is the diffusion coefficient of Na taken here as 1.23×10^{-5} cm^2./sec., and r the radius is 0.088 cm. By a method of successive approximations the above equation is satisfied when $D\pi^2 t/r^2 =$ 0.3 and $t = 19$ seconds.

of a frog egg. Assuming no retardation at the surface of the object and a diffusion coefficient of 1.23×10^{-5} cm^2/sec. internally, the time required for half of the sodium to exchange is 19 seconds. In the case of the actual egg, the time required for half of the exchangeable sodium to exchange is 15 minutes. The exchange of sodium in the egg may be slower for at least two reasons: delay in passing through the cell membrane, and the existence of a low diffusion coefficient within the egg. The fact that we have obtained "ring" radio-autographs after exposure of five minutes is explainable only on the basis that the movement of sodium in the egg is much slower than in Ringer solution, and that the membrane has little limiting effect on the exchange.

On the assumption that the membrane presents no barrier, one can calculate a value for the diffusion coefficient within the egg. Thus, for $Q/Q^{\infty} = 1/2$, $D\pi^2 t/r^2 = 0.3$, and $D = 2.6 \times 10^{-7}$ cm^2./sec.

From the curves given in Figures 3 and 5, it is clear that only 12 per cent of the sodium in the ovarian frog egg is readily available for exchange. Part of the remaining sodium can be exchanged over a period of many hours. Since the factors which govern this slow exchange are wholly within the egg itself, some form of internal blocking must occur. Speculation concerning the mechanism is still unwarranted. We believe that the finding that 12 per cent of the sodium behaves differently from the remainder is of considerable importance to others who are performing tracer experiments. In this experiment, the behavior of the traced substance was not characteristic of the behavior of all the sodium of the cell. Upon the other hand, it would appear that the difficulty raised might be turned into an advantage. One has a technique for studying the various degrees of binding and the chemical activity of cellular components.

It is of special interest that sodium could be traced into the germinal vesicles. Our experiments thus show that the nuclear membrane is permeable to sodium. They also show that sodium is normally present in a cell nucleus. It is especially significant that the concentration of this cation in the nucleus is at least double that of the cytoplasm. The relatively large amount of active sodium found in the nucleus may be connected with the fact that the nucleus has a much higher percentage of water than does the yolk cytoplasm.

Our data on the increased permeability of dead cells to Na^{24} offer a basis for explaining some of the empirical observations of Quimby (1947). Working with second and third degree burns she was able to show that the more severely damaged tissues took up much greater amounts of tagged sodium. These results must be due to the presence of dead cells. Furthermore, she found that addition of hypertonic saline materially aided recovery. It is clear, therefore, that in both sets of observations a fundamental factor is operating. This epitomizes, in the case of sodium, what many physiologists have long believed to be a basic diagnostic character of life—namely, that a living cell can discriminate between ions both quantitatively and qualitatively in its extracellular environment and can build specific internal gradients and unequal distributions, which a dead cell certainly cannot do. As we have shown in the case of the amphibian egg, permeability to radioactive sodium can be used as an indication of life or death in a cell.

Summary

1. The ovarian egg of the frog *Rana pipiens* is freely permeable to Na^{24}.

2. At least two different types of binding limit the internal diffusibility of sodium within the egg. Only 12 per cent of the normal sodium is readily exchangeable. The remainder exchanges very slowly.

3. The implications of finding non-equilibration of such a simple ion as sodium are presented.

4. A new technique for making radio-autographs of single cells shows that after half an hour sodium is distributed almost uniformly throughout the cytoplasm.

5. Calculations based on rate of exchange of sodium into the egg plus radio-autograph evidence give a value of 2.6×10^{-7} cm^2./sec. for the diffusion coefficient of sodium within the egg.

6. At equilibrium the nucleus possesses approximately twice as much traced sodium per unit volume as the cytoplasm.

7. By direct chemical analysis the sodium content of frog eggs was found to be 0.082 per cent of the wet weight.

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STUDIES IN THE REGULATION OF BLOOD-SUGAR CONCENTRATION IN CRUSTACEANS. I. NORMAL VALUES AND EXPERIMENTAL HYPERGLYCEMIA IN LIBINIA EMARGINATA

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INTRODUCTION

Studies of blood-sugar concentrations of invertebrates were, until relatively recently, confined for the most part to defining the range of glycemic values in different species. Stimulated by investigations of the effects of insulin on blood-sugar concentrations in mammals, these early studies among invertebrates demonstrated that considerable variations existed in the amount of glucose in the blood, even within a single species. The reviews of Beutler (1939) and of Benazzi-Lentati (1941) summarize many of these observations.

The wide range of glycemic concentrations in crustaceans reported by different investigators, who at times studied the same species, was soon recognized to be due, in part, to the different analytical methods employed and, perhaps even to a greater extent, to the varied physiological states of the animals at the time blood samples were taken for analysis. This latter possibility led to observations on animals maintained under more critical laboratory conditions and to studies of factors that influenced the amount of sugar in the blood.

Thus, where Hemmingsen (1924a) had found an increase in blood sugar in Astacus after feeding, Kisch (1929) reported a decrease in *Carcinus maenas* during starvation. These results were confirmed both by Stott (1932) and by Florkin (1936) for the same species of Carcinus. On the other hand, Roche and Dumazert (1935) found that the blood glucose of *Cancer pagurus* starved for one month did not differ significantly in concentration from that of freshly captured individuals. Asphyxiation was reported by Stott (1932) to cause marked hyperglycemia in *C. pagurus, Portunus puber,* and *Carcinus maenas;* this observation was confirmed by Roche and Dumazert (1935) on *Cancer pagurus.* Stott (1932) also observed a high concentration of glucose in the blood of newly-molted crustaceans (within a few hours of ecdysis) compared with animals before the molt.

Hemmingsen's studies (1924b) on the crayfish Astacus led him to believe a regulatory mechanism was present for maintaining a constant level of blood sugar. The basis for this view was his observation that samples of concentrated glucose solution when injected into Astacus disappeared from the blood stream too rapidly to have been oxidized to CO_2 during the experimental period, and yet no glucose was excreted in detectable amount into the water in which the injected animals

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were maintained. These results Hemmingsen regarded as evidence for a hypoglycemic regulatory mechanism.

In addition, a number of pharmacological substances had been reported as being effective in inducing hyperglycemia in crustaceans. The controversy in this branch of the general problem lay not so much in the interpretation of such hyperglycemias when they occurred, but whether these substances really induced hyperglycemia. Thus, Medvédéva (1936) reported that injection of adrenalin in Potamobius (Astacus) caused hyperglycemia, but that injection of insulin was without any definite effect. Roche and Dumazert (1935), on the other hand, reported that neither adrenalin nor insulin had any appreciable effect on the blood-sugar concentration of *Cancer pagurus*. Kalmus and Waldes (1936) stated that not only adrenalin and insulin, but also such non-specific substances as hydroquinone and sodium chloride solution, effected marked hyperglycemias when injected into crayfish. Florkin and Duchateau (1939), using more carefully controlled procedures, reported that insulin had no effect while adrenalin produced a hyperglycemia in the crayfish, thus confirming the observations of Medvédéva.

These scattered observations, controversial though they may have been, indicated the possible existence of a hyperglycemic mechanism, just as Hemmingsen's studies had indicated the possibility of a hypoglycemic mechanism. The first indication of a definite anatomical structure which might be involved in regulating sugar metabolism in crustaceans was made by Abramowitz, Hisaw and Papandrea (1944). These authors found that injection into *Callinectes sapidus* of aqueous extracts of crustacean eyestalks increased the concentration of blood sugar within an hour. More specific localization of the source of this diabetogenic factor was demonstrated by the preparation and injection of extracts prepared from the sinus glands of Hanström that had been removed from eyestalks. The injection of such extracts resulted in a marked hyperglycemia amounting to nearly four times the normal basal concentration of blood glucose. When extracts, prepared from the remainder of the eyestalks from which the sinus glands of Hanström had been previously removed, were injected, they were practically without hyperglycemic effect. These investigators' complementary experiments, which consisted of removing the sinus glands by ablation of both eyestalks, to determine whether hypoglycemia would ensue, gave negative results; in fact, over a period of seven days after eyestalk removal, there was an anomalous, slight increase in concentration of blood sugar.

Our own studies were undertaken to define in greater detail the nature of such glycemic changes under experimental conditions and to investigate the physiology of the regulatory processes. This is the first report in detail of our investigations, some of which have appeared in abstract form (Kleinholz, 1948; Kleinholz and Little, 1948; Kleinholz and Havel, 1948).

Materials and Methods

The animals used in this study were the marine spider crab, *Libinia emarginata*. A large stock of animals was maintained by the laboratory collectors in a live-car. Other than the occasional placing of a freshly killed fish into the live-car, no regular feeding of these stock animals was undertaken. When groups of animals were removed for use in the laboratory, they were starved for three days before

blood samples were taken, to insure a basal level of blood-sugar concentration, and were not otherwise fed except where indicated. Such experimental animals were marked for identification by painting serial numbers with lacquer on the dorsal surfaces of the carapace of each; the crabs were then placed in individual containers, similarly numbered, through which a stream of sea water circulated. It was hoped that the hyperglycemic effect of crowding reported by Abramowitz et al (1944) would be reduced or obviated by such isolation. Only male individuals were used in this study.

Tuberculin hypodermic syringes of 1 ml. capacity, graduated in hundredths of a milliliter, were used for taking blood samples. Each syringe was calibrated to deliver 0.5 ml. by weighing the volume of water delivered between the 0.60 ml. and 0.10 ml. marks on the barrel of the syringe. Blood for analysis was taken from the sinuses of the walking legs, the arthroidal membrane between the base of the leg and the body being first wiped dry with filter paper or absorbent cotton. The syringe was filled slightly beyond the 0.60 ml. mark and, after withdrawing the needle from the sinus, was emptied to this mark, the excess droplet of blood being removed by touching the tip of the needle to filter paper. A 0.5 ml. sample of blood could thus be delivered into tubes containing the deproteinizing mixture. To avoid injury to the arthroidal membrane that would ensue from the repeated bleeding of the same individual, samples were taken from different legs on both sides of the animal. In all of the experiments reported here, blood samples from control and from experimental animals were taken in the daytime, in most cases in the forenoon. The possibility of a diurnal variation in concentration of blood sugar was thus avoided.

It was found most convenient to work with groups of six Libinia at a time. In most instances blood samples were taken from the individuals of a group before a particular treatment, and then again after the experimental treatment, each crab thus serving as its own control. The control blood samples and the experimental blood samples were carried through the analytical procedure simultaneously and received comparable handling. The method for determining the amount of blood glucose was that described by Miller and Van Slyke (1936). It is reported that this method, when used with mammalian blood, gives "true" blood-sugar values, which do not include non-fermentable reducing substances. We have found a significant amount of non-fermentable reducing substance present in Libinia blood, so that in our hands the method must be considered as expressing *total reducing substances* as glucose equivalents. The procedure was essentially as described by Miller and Van Slyke. The dilute ceric sulfate for the titration was prepared fresh daily from the stock solution.

Two blanks, consisting of the reagents used in the glucose determination, were used with each set of blood samples. These blanks required about 0.15–0.25 ml. of the dilute ceric sulfate to reach the same end-point obtained in the titration of the blood samples. This wide range was due to the preparation of a second lot of stock reagent solutions during the course of the work. For the first set of stock solutions the blanks varied from 0.18–0.25 ml., the average for 38 blanks being 0.22 ml.; with the second set of stock solutions the blanks varied from 0.13–0.18 ml., the average for 20 blanks being 0.15 ml. The average for the total 58 blanks was 0.195 ml. of the dilute ceric sulfate.

The accuracy of the Miller and Van Slyke method in our hands was tested by determining the amount of glucose in prepared solutions of known concentration. These concentrations ranged from 16–200 mg. of glucose per 100 ml. of solution. The average percentage of error for 13 such determinations was ± 3.9 per cent.

Where Libinia without eyestalks were used to determine the effect of absence of the sinus glands on the blood-sugar level, bilateral eyestalk ablation was done with the aid of fine dissecting scissors. Bleeding from the cut surface was very slight and ceased upon the formation of a blood clot in the orbit.

The distinction between total reducing substances and non-fermentable reducing substances in the blood was made by fermenting one of two blood samples with a 10 per cent suspension of Fleischmann's yeast. The yeast was prepared by suspension in distilled water, centrifuging, and pouring off the supernatant. After three such washings, the final 10 per cent suspension was kept in the refrigerator until used. In the fermentation, 3.5 ml. of the yeast suspension and the 0.5 ml. blood sample were mixed and allowed to remain at 22° C. for one hour, after which the mixture was centrifuged and the supernatant poured off into a second tube containing acid cadmium sulfate. The yeast and blood residue was similarly washed and centrifuged three times with 1 ml. portions of distilled water, the supernatants each time being added to the first one. A second 0.5 ml. blood sample taken from the same animal had been prepared for the routine analysis. Both blood samples were carried through the analytical procedure simultaneously, using adequate blanks (washings of a 3.5 ml. aliquot of yeast suspension) for the fermented samples.

The reliability of this method of fermenting glucose in blood was tested with samples from six Libinia. To a 0.5 ml. portion of blood from each animal was added 0.5 ml. of solution containing 5.06 mg. of glucose (thus equivalent to adding 1012 mg. per cent of glucose to the blood sample) and 3.5 ml. of the 10 per cent yeast suspension. The blood samples were fermented and treated as described above. The glucose-equivalent in reducing substances present in these samples ranged from 6.3–11.1 mg. per cent, with an average of 8.9 mg. per cent, showing practically complete fermentation of the added glucose.

Observations

A. Normal and eyestalkless animals

The studies of Abramowitz, Hisaw and Papandrea (1944) pointed to the sinus glands as being mediators in the hyperglycemic response following injection of prepared extracts. Their attempts to observe whether removal of this gland (by ablation of both eyestalks) resulted in hypoglycemia yielded paradoxical results, a gradual hyperglycemia being observed in such animals over a period of seven days.

Our own observations made over a longer period in a comparable series of experimental animals, do not confirm the latter results of these investigators. Of eighteen animals brought into the laboratory at the same time, the eyestalks of twelve were ablated, while the remaining six served as normal control animals. The animals of the control group were isolated in individual containers; six of the operated Libinia were designated as Group A and were similarly isolated, while the remaining six operated animals, constituting Group B, were placed in a com-

mon tank. These animals were not fed during the time the experiment was in progress. Beginning on the morning of the third day after eyestalk removal, blood samples were taken from the individuals of Group A and of the control group; on the following morning, the fourth day after ES removal, samples were taken for analysis from individuals of Group B. By alternating in this fashion and taking blood samples every third day, the observations were extended over a period of twenty-six days. The results which are shown in Table I are the averages and the standard deviations for the six animals constituting each group.

TABLE I

Comparison of the blood-sugar concentrations in two groups of Libinia without eyestalks, with that of a normal control group. The figures are the averages for the 6 crabs of each group and their standard deviations.

Days after eyestalk removal	Blood-sugar concentration in mg.-per cent		
	Group A	Group B	Control
3	10.1 ± 2.5	—	6.9 ± 1.2
4	—	11.1 ± 2.1	—
6	12.0 ± 3.1	—	9.9 ± 1.5
7	—	9.0 ± 2.5	—
10	10.2 ± 3.4	—	7.4 ± 1.3
11	—	9.3 ± 3.6	—
17	10.2 ± 4.3	—	7.3 ± 1.8
18	—	9.6 ± 3.7	—
24	10.1 ± 3.2	—	8.7 ± 1.7
26	—	7.9 ± 2.6	—

As might be expected, there were variations in the concentration of blood sugar not only among the individuals of a group, but also in the same individual at the different intervals when blood was taken for analysis. These variations are probably due to a combination of actual fluctuations in glucose concentration and of slight artifacts in the analytical procedure. The relatively low average of the blood-sugar concentration in the control group on the third day, compared with later averages, is probably to be explained on this basis, for the average glycemic values determined for three different normal groups, which had been starved three, six, and ten days, were, respectively, 7.9, 8.3 and 9.1 mg. per cent. We conclude from the data of Table I that removal of the sinus glands has no marked effect on the basal level of the blood-sugar concentration. The hyperglycemia reported by Abramowitz et al. after eyestalk removal in Callinectes could not be confirmed with Libinia.

B. Total reducing substances and true blood sugar

It has been known that the blood of mammals contains, in addition to glucose, other reducing substances which, in the analytical methods currently employed, may contribute significantly to the total value obtained as "apparent" glucose. For an accurate measure of the amount of glucose in a blood sample the supple-

mentary use of a yeast fermentation method along with the conventional determination permits the distinction to be made between total reducing substances or apparent blood glucose, and non-fermentable reducing substances; the difference between the two such determinations is then considered to represent the fermentable glucose.

The application of methods devised for the analysis of mammalian blood to that of invertebrates would require similar supplemental yeast fermentation methods, since little is known about the presence or the nature of non-fermentable reducing substances in blood of the latter group. Such yeast fermentation analyses of the blood of Libinia, using the procedure described under "Methods," were conducted in parallel with samples taken at the same time for the determination of total reducing substances. The results for experimental and control animals are arranged in Table II.

TABLE II

True blood-sugar concentrations in groups of eyestalkless and of normal Libinia. Each group consisted of 6 animals. TRS, *total reducing substances;* NFRS, *non-fermentable reducing substances;* TBS, *true blood sugar. Figures are averages for the animals of a group and the standard deviations from the mean.*

Animals and condition	Concentration in mg. per 100 ml. blood		
	TRS	NFRS	TBS
Group A, starved 24 days	10.1 ± 3.2	7.4 ± 0.8	2.7
Group B, starved 26 days	7.9 ± 2.6	5.0 ± 1.3	2.9
Controls, starved 24 days	8.7 ± 1.7	6.8 ± 1.7	1.9
Normal, starved 6 days	8.3 ± 1.3	6.3 ± 0.7	2.0

The data shown in this table represent the averages for four groups of Libinia, each group consisting of six animals. Three of these groups consisted of individuals whose blood analyses for total reducing substances had been made at intervals over nearly four weeks, as shown in Table I. The animals of Group A, Group B, and the control group are described in the text above and in the preceding table. On the twenty-fourth and twenty-sixth days when final analyses were being made on these three groups, additional samples were taken at the same time for determination of the non-fermentable reducing substances after the blood sample had been mixed with yeast suspension and fermented. The fourth group of Table II consisted of normal animals which had been starved six days, in comparison with the twenty-four days of starvation undergone by the control group to the eyestalkless condition. The figures which are given for concentration of true blood sugar are averages for the six individuals of a group, representing the differences between the average concentrations of total reducing substances present in one set of samples and the average amounts of non-fermentable reducing substances found in similar samples after they had been fermented by the yeast suspension.

In all groups the amount of true blood sugar is quite low. There appears to be no difference in glycemic level between normal animals starved for a short period (6 days) and those starved for an appreciably longer time (24 days). At

first glance the slightly higher level of true blood sugar in the groups of eyestalkless individuals as compared with that in the normal control animals might seem to indicate an alteration in glucose metabolism as a result of eyestalk removal, but in view of the small number of animals involved in the experimental groups and the comparatively high standard deviations of the averages for each group, it is doubted that these differences from the controls can be regarded as significant.

C. *Hyperglycemia as a result of injection of eyestalk extract*

The hyperglycemic effects obtained by Abramowitz, Hisaw and Papandrea (1944) in Callinectes upon injection of eyestalk extracts showed a rough agreement between dosage and the increment of the resulting hyperglycemia. But since their determinations were in terms of total reducing substances, with no distinction being made between fermentable and non-fermentable components, closer examination was made of these components of the total reducing substances at the same time that we tried to confirm their observations.

Blood samples from a group of six Libinia from which both eyestalks had been ablated were analyzed on the first and fifteenth days after eyestalk removal. At these times the average concentrations of total reducing substances were respectively 7.1 ± 1.5 mg. per cent and 6.3 ± 1.6 mg. per cent, confirming previous observations made in Table I that no significant change follows in the glycemic level of animals from which both eyestalks have been removed. On the morning of the seventeenth day after eyestalk ablation, each of the crabs was injected with 0.1 ml. of extract prepared from the eyestalks of Libinia, so as to receive the equivalent of one eyestalk. One hour after the injection, blood samples were taken from each animal for determination of both the total reducing substances and the non-fermentable reducing substances. Five of the six injected Libinia showed striking increments in total reducing substance in the blood, the concentrations after injection being from twice to nearly five times those obtained before injection; in the sixth animal the amount of total reducing substance after injection was about 60 per cent greater than before the injection. The average value for total reducing substance for all six animals was 17.3 ± 6.4 mg. per cent. The average for non-fermentable reducing substances of the post-injection samples after yeast treatment was 4.5 ± 0.4 mg. per cent; average fermentable blood sugar after injection was therefore 12.8 mg. per cent. Yeast fermentations were not made on blood samples taken on the first and fifteenth days after eyestalk removal, but if the average value for true blood sugar is assumed to be comparable to those shown in Table II, then the average increase in fermentable blood sugar after injection of eyestalk extract is well over 400 per cent. We are thus able to confirm the observation of Abramowitz et al. that injection of crustacean eyestalk extract induces a marked hyperglycemia in crustaceans, and to show, furthermore, that this increase is apparently a fermentable sugar.

D. *Hyperglycemia as a result of asphyxia*

Stott (1932) had reported a large increase in blood sugar of crustaceans which had been kept for ten hours in containers of sea water that had been tightly covered. This change he attributed to asphyxia due to the decrease in oxygen content of the

water during the period of the experiment, because when an adequate air supply was again made available to the animals by removing the cover, the blood-sugar concentration returned to the normal level. Roche and Dumazert (1935) confirmed this observation by reporting that removing animals from sea water and keeping them in air for 30–60 minutes resulted in a marked hyperglycemia. Both studies reported this hyperglycemia as a direct observation, with no attempt to investigate in further detail the mechanism of this response.

Similar results were obtained by us with Libinia, and a possible mechanism for what we shall call the hyperglycemia of asphyxia was indicated by further study. In these experiments, groups of six normal and six eyestalkless animals were employed. To obtain partial asphyxia during which the animals could be kept under observation, the method of Roche and Dumazert was used: removing the animals from sea water and keeping them in air for 60 minutes, and then removing a blood sample from each for analysis and for comparison with samples before asphyxia. The results of these experiments are shown in Table III.

TABLE III

Effect of asphyxia on the blood-sugar concentration of normal animals and animals without sinus gland

Animal group	Eyestalk condition	Days starved	Concentration in mg.-per cent	
			Before asphyxia	After asphyxia
Group B Nos. 13–18	ES off 31 days	31	(7.9 ± 2.6)*	7.0 ± 2.8
Controls Nos. 19–24	Normal	31	(8.7 ± 1.7)*	16.1 ± 8.3
Nos. 31–36	ES off 1 day	18	7.1 ± 1.5	6.0 ± 1.1
Nos. 25–30	Normal	19	7.3 ± 1.0	22.0 ± 12.2

* See Table I.

The first two groups of crabs tested consisted of eyestalkless individuals which had been under observation for several weeks (the animals constituting Group B of Table I), and a similar number of normal Libinia which had been their controls. Both groups were removed from sea water and placed in individual finger-bowls in air. After one hour of such exposure, blood samples were taken for analysis (at this time the animals were limp and showed a marked loss of muscular tone; following their return to sea water recovery was rapid). No blood samples were taken in this experiment directly before the asphyxiating experience, the glycemic values which had been determined at regular intervals for the preceding twenty-six days being considered sufficient to serve as a standard. As can be seen from Table III, the effect of this asphyxia was different in the two groups, the crabs without eyestalks showing no appreciable change in their average concentration of blood sugar, while the group of normal animals showed a marked increase in the glycemic average for the group.

The experiment was then repeated with two additional groups of similar animals. This time a blood sample was removed before subjecting the animals to asphyxia, and the second sample was taken immediately after the 60 minutes of asphyxia. The results were similar to those obtained previously; the normal animals (with eyestalks) showed a marked hyperglycemia, the average concentration being three times the pre-asphyxia level, while the animals without eyestalks showed no significant change from the glycemic level before asphyxia. These results show that the observed hyperglycemia is dependent upon the intact eyestalk and indicate the possibility that the response may be mediated by the sinus gland. The results of more exact studies, in which sinus glands were removed from otherwise intact eyestalks, to define the mechanism of the hyperglycemic response, will be reported later.

E. Alimentary hyperglycemia

A number of investigators have reported the effects of feeding and inanition upon blood-sugar levels in crustaceans. The studies of Hemmingsen (1924a) and Stott (1932) showed that feeding resulted in a rise in blood-sugar concentration. Stott had found that in a group of starved *Carcinus maenas,* the glycemic level ranged between 5–8 mg. per cent. When such animals were fed mussels, the blood sugar rose to values of 20 mg. per cent or more over a period of several hours; about fourteen hours after such feeding, the level of blood sugar returned to a concentration of approximately 5 mg. per cent.

In view of the part played by the eyestalk and sinus gland in mediating the hyperglycemia resulting from asphyxia, as described in the preceding section, it was thought advisable to determine whether alimentary hyperglycemia was similarly regulated. Seven Libinia from which both eyestalks had been removed three days previously, and which had been starved for three days, were isolated in individual containers. The average glycemic value immediately before feeding was 9.1 ± 2.8 mg. per cent. Each animal was then supplied with 5–10 grams of the visceral mass of *Venus mercenaria,* which was devoured within fifteen minutes. Blood samples taken three hours after this feeding showed a marked rise in sugar content in each of the seven animals, the average for the group after feeding being 18.3 ± 4.7 mg. per cent. The results therefore indicate that alimentary hyperglycemia is not mediated by the sinus glands in the eyestalks.

Summary

1. Removal of the sinus glands by eyestalk ablation in unfed *Libinia emarginata* has no significant effect on the blood-sugar concentration when compared with similarly unfed controls.

2. Values for true blood sugar, as distinguished from total reducing substances, were determined after yeast fermentation of blood samples. In starved animals the concentration of total reducing substances is between 8–9 mg. per cent; that of non-fermentable reducing substances, 6–7 mg. per cent; that for true blood sugar is therefore about 2 mg. per cent.

3. Injection of eyestalk extract increases the concentration of total reducing substances in the blood. This increase is in the fermentable component, amounting

to over 400 per cent of that in the uninjected animal, and therefore probably represents a true hyperglycemia.

4. Asphyxia also causes hyperglycemia, the total reducing substances in blood samples being two to three times the concentration preceding asphyxia.

5. Removal of the sinus gland by eyestalk ablation prevents the appearance of the hyperglycemia of asphyxia. The sinus gland may be a mediator in certain hyperglycemic responses of crustaceans, but does not seem to be concerned in alimentary hyperglycemia.

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OVARIAN INHIBITION BY A SINUS-GLAND PRINCIPLE IN THE FIDDLER CRAB

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The action of the sinus gland in inhibiting ovarian development was first demonstrated by Panouse (1943, 1944, 1946) working with females of the shrimp, *Leander serratus*. In these animals, amputation of both eyestalks or bilateral removal of the sinus glands resulted in a great acceleration of ovarian growth, maturation of the oöcytes, and even laying of mature eggs, during a period when these structures are normally quiescent or just beginning the normal growth phase. Implantation of sinus glands into abdomens of destalked animals resulted in an inhibition of ovarian development. Similar results following eyestalk removal were obtained with the crayfish *Cambarus immunis* by Brown and Jones (1947).

The following experiments were performed upon the fiddler crab, *Uca pugilator*, to ascertain whether this phenomenon of ovarian inhibition by a blood-borne principle from the sinus glands also obtained in the division, Brachyura, of the Crustacea.

Materials and Methods

The animals used in the experiments were females of *Uca pugilator* collected neâr Woods Hole, Massachusetts, on July 10, 1948. The carapace widths ranged from 15 to 20 mm. at the widest point. They were kept in the laboratory at room temperature (about 25° C.) in individual containers each holding sea water to a depth of a quarter of an inch. The water was changed daily. The animals were not fed during the course of the investigation.

Removal of eyestalks was accomplished by amputation at their bases and the wounds were allowed to close spontaneously by clotting of the blood which welled slowly from them.

Sinus glands were obtained from donor animals for the purpose of implanting according to the following procedure. Eyestalks were removed as above, placed in sea water, and the contents of the eyestalks exposed by a dorsal splitting of the chitinous sheath. The sinus gland, a discrete bluish organ, was dissected free of surrounding tissue and drawn into the lumen of a 25 gauge needle by means of a tuberculin syringe. The gland, plus a minute quantity of sea water, was injected into the ventral hemocoele of the recipient animal's abdomen. The chitinous membrane of this region of the body is transparent and thus it is possible to see the actual extrusion of the contents of the needle. The dissections and implantations were accomplished with the aid of a dissecting microscope.

[1] This investigation was supported by a research grant from the graduate school of Northwestern University.

In order to observe the influences of the above procedures, experimental and normal control animals were autopsied as they died, or were sacrificed for dissection at six-day intervals. The carapace and hypodermis were removed, and the ovary, an H-shaped organ lying over the hepatopancreas and just below the hypodermis, was dissected out in sea water and placed in a tared watchglass for weighing. Excess moisture was removed with filter paper, and fresh weights were taken.

Results

On July 11, 1948, ten normal animals were sacrificed and the ovaries removed. Eight of these ovaries were found to be in an immature state; the oöcytes were very small and the organs as a whole were slender and of a light yellow-pink color. The other two ovaries contained somewhat larger oöcytes and the color of the organ was a deep shade of pink. The average of the ten ovarian weights was 12.6 mg., the extremes being 6.8 mg. and 18.8 mg. respectively

On July 12, 120 animals of nearly uniform size were selected and divided into three lots. Eyestalks were removed from two lots of forty of them. Two days later, one sinus gland was implanted into each of one lot of forty of the eyestalkless animals using the technique described earlier. The implants were then repeated every fifth day for the duration of the experiment.

TABLE I

Number of specimens, and ranges and averages of ovarian fresh weights in milligrams

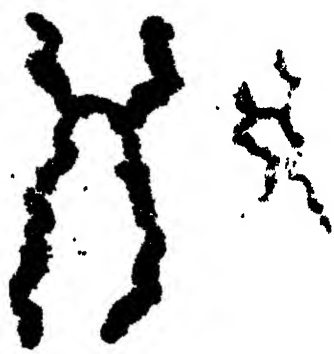
Days	Destalked			Destalked, receiving sinus gland implants			Controls		
	No. spec.	Range	Av.	No. spec.	Range	Av.	No. spec.	Range	Av.
1–6	6	11.8–46.2	24.0	14	7.2–45.2	20.6	6	9.2–16.2	13.5
7–12	4	17.9–61.2	32.6	10	6.4–67.6	24.7	4	6.6–13.1	10.1
13–18	5	32.6–54.9	42.2	4	12.5–35.8	21.0	4	4.2–34.2	18.4
19–24	11	23.0 165.6	54.7	5	8.8–32.3	19.5	11	5.8–23.6	13.9
25–30	6	33.8 160.4	66.4	4	15.8–22.3	17.9	6	4.4–19.4	11.0

In Table I are summarized the ovarian weights of the three groups of animals (destalked, destalked and receiving sinus gland implants, and normal controls) which died or were sacrificed during five succeeding six-day periods. Each value obtained represents the average of data from 4 to 14 animals. It will be observed from Table I that over the thirty-day period the average ovarian fresh weights of the destalked animals increased approximately linearly with time from an original 12.6 mg. to 66.4 mg., a more than five-fold increase. The ovarian weights of destalked animals receiving sinus-gland implants showed an initial rise, with an approximate level being maintained at values somewhat ~~higher~~ (a total average of 7 mg.) than those of the control animals.

Other changes observed in the ovaries of the destalked animals, in addition to the gross size alteration, were gradual increases in oöcyte diameter and a shift of their color from the previously mentioned pinkish-yellow to a deep purple-red. The color change became most striking as the ovary attained a weight of approximately 15–18 mg.

In general, the color and size of oöcytes of the destalked animals receiving sinus-gland implants were found to lie somewhere between the extremes offered by the destalked and control animals.

During the course of the experiment, only 7 of the 31 control animals were found at autopsy to have ovaries in the apparently mature condition typical of the destalked ones. On the other hand, after the first six-day period, in no case did any of the latter group contain oöcytes presenting an immature appearance, either in size or in color.



A B

FIGURE 1. Ovaries of two fiddler crabs removed September 1, 1948: A, from an animal destalked one month earlier: B, from a normal animal.

Figure 1 is a photograph of two ovaries removed from animals of the same carapace width—17 mm. The animal from which ovary A was removed was destalked on July 30, 1948, and sacrificed for dissection on September 1, 1948. The animal from which ovary B was removed was a normal control maintained under identical laboratory conditions during the same period and sacrificed on the same day. These two organs are typical of those removed from destalked and normal animals, respectively, during the course of the experiment. The approximate wet weights are: ovary A, 80 mg.; ovary B, 10 mg.

It is also of interest to note that during the time that the investigation was in progress, five females which had been deprived of eyestalks and received no sinus-gland tissue laid mature eggs, and one female, also eyestalkless, which had received one sinus gland implant, did likewise. In none of these cases were the

eggs fastened to the pleopods of the animal as normally occurs. No eggs were laid by any of the control animals.

Discussion

It seems apparent from the foregoing results that the sinus gland in Uca, as in Leander and Cambarus, is the source of an ovary-inhibiting principle which, when absent, allows for a period of ovarian growth and development even at a time when no such gonadal activity would otherwise be manifested.

Implantation of the quantity of sinus-gland tissue utilized in this work into the abdomens of destalked animals tends to suppress the gonadal growth, but allows the ovary to be maintained at a stage somewhat more mature than that characteristic of the normal animals in possession of both sinus glands.

There is no indication from these experiments whether the principle from the sinus glands inhibits the ovary directly or serves to inhibit the production of a gonad-stimulating principle normally produced elsewhere in the body. On the latter hypothesis one possible explanation of the ovarian growth during the first six-day period in the gland-implanted animals is that during the two days elapsing between eyestalk (sinus gland) removal and the first implant, the blood titer of the inhibitor dropped to such a point that a gonad-promoting principle was permitted to be liberated into the blood. The first implant might be presumed to inhibit further production of the stimulating principle but not counteract the action of this factor already present.

An explanation based upon an hypothesis of a direct inhibition of ovarian growth is as follows: During the two days which elapsed between amputation of the eyestalks and the initial implantation of the sinus-gland tissue, there was a drop in titer of the inhibitory substance to an ineffectual level which permitted nearly as rapid growth in these ovaries as occurred in the destalked animals which received no implanted sinus glands. There may also be a delay in the production of an inhibiting concentration by the implants.

There is a suggestion in Table I that sinus-gland implants in the eyestalkless animals not only are able to inhibit growth in the partially developed ovaries, but may even effect a reduction in their size.

Summary

1. Removal of the eyestalks of adult females of *Uca pugilator* results in a period of rapid ovarian growth in which the increase in fresh weight of the gonad is approximately five-fold in a thirty-day period.

2. The period of ovarian growth is characterized by increase in oöcyte diameter and a color change from light pink to a deep purple-red.

3. Implantation of sinus-gland tissue into the abdomens of destalked females serves to inhibit to a large degree this rapid growth.

4. Six of the animals which had been deprived of their eyestalks laid mature eggs during the course of the experiment; none of the controls did so. Eggs produced by the experimental animals failed to become attached to the pleopods.

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PIPERAZINE DIHYDROCHLORIDE AND GLYCYLGLYCINE AS NON-TOXIC BUFFERS IN DISTILLED WATER AND IN SEA WATER [1, 2]

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A wide selection of buffers is necessary in biological work, since it is often desirable to repeat a particular experiment with a different buffer. Piperazine dihydrochloride and glycylglycine are crystalline, non-volatile, very soluble solids readily obtainable in pure form. Piperazine is relatively non-toxic to man (Hanzlik, 1917) and to rats (Dieke, Allen, and Richter, 1947) and has been used as an apparently non-toxic buffer by certain biologists at our suggestion (Cornman, 1940, 1941; Evans, Beams, and Smith, 1941). The buffer merits of glycylglycine in sea water have been previously pointed out by Tyler and Horowitz (1937). This relatively non-toxic material is a normal constituent of many proteins. A wide-range buffer is simply and accurately prepared from only these two substances and sodium hydroxide. Used in sea water there is no observed precipitation of salts until a pH of 9.9 is reached. The commonly used phosphate buffer precipitates calcium and magnesium phosphate from sea water at a much lower pH, thus disturbing the salt balance and adding uncertainty to conclusions from experiments. The shortcomings of many of the buffers in common use have recently been mentioned by Gomori (1946). We have not used the new buffers suggested by him and cannot compare his buffers with ours, except to point out that our buffers have a wider range.

For special cases where it is desired to have no inorganic ions in a buffer, it is possible to obtain buffers from a pH of 7.0 to 11.0 by titrating glycylglycine with the free base of piperazine. However, we are presenting no data on this subject.

In this paper we present a table indicating the preparation of several buffers, using piperazine dihydrochloride, glycylglycine and equimolecular mixtures of the two substances in distilled water and in sea water. We also present pK_1 and pK_2 values of piperazine dihydrochloride.

Experimental

The piperazine was purchased from the Eastman Kodak Co. in the form of the hexahydrate. Because the free base of piperazine absorbs carbon dioxide and moisture from the air, it was converted into the stable dihydrochloride (Sieber, 1890) before use.

[1] A brief report of this work was presented at the Florida Academy of Science Meeting, Tampa, Fla., December 1946.

[2] We are indebted to Dr. W. Mansfield Clark for certain suggestions relative to the manuscript, and to H. G. Smith for help with the experiments.

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Piperazine dihydrochloride is prepared by dissolving 50 g. of piperazine hexahydrate in 100 ml. of 95 per cent ethanol, and adding slowly 100 ml. of concentrated hydrochloric acid. Heat is evolved. As the mixture cools, crystals of the dihydrochloride hydrate are formed. The mixture is cooled in an ice bath and is filtered. The crystals are washed several times with cold ethanol, and are air-dried. The material is ready for use after it has been dried at 100° C. for eight hours. The yield is 33 g. Anhydrous piperazine dihydrochloride is slightly hygroscopic.

Analytically pure glycylglycine was purchased from the Amino Acid Manufactures of the University of California at Los Angeles and was used without further purification. The material was dried at 100° C. for six hours just prior to use. Glycylglycine is not appreciably hygroscopic.

The pH measurements were made at 25° C. (± 0.2) with a Leeds and Northrup potentiometer-electrometer No. 7660, equipped with Leeds and Northrup glass dip electrode Std. 1199–12 made of Corning 015 glass, and a reference saturated calomel half-cell electrode Std. 1199–13 with a potassium chloride capillary salt bridge. Before and after each titration the electrode was checked against "standard acetate," for which the pH value of 4.64 was taken (MacInnes, Belcher, and Shedlovsky, 1938). As is the practice in standardizing buffers, the liquid junction potentials were neglected.

The sodium ion error for the higher pH values was corrected by using the following equation adapted from Powney and Jordan (1937) to fit the sodium ion errors found experimentally when our glass electrode was calibrated with the hydrogen electrode.

$$\text{Log } \Delta\text{pH} = 0.50 \text{ pH} - 5.86 + 0.46 \log [\text{Na}^+]$$

The possible error in these readings increases with increasing alkalinity, but below a pH of 9.0, the accuracy was within the limits of ± 0.02.

Although stock solutions of piperazine dihydrochloride and glycylglycine may be prepared, it is preferable to prepare the solutions fresh, since on long standing glycylglycine may undergo hydrolysis and piperazine dihydrochloride might form toxic products (Greenbaum, 1937).

Solutions of piperazine and glycylglycine and equimolecular mixtures of the two were titrated with standardized sodium hydroxide and numerous readings were taken. From these readings a table for the preparation of buffers was made (Table I).

Several pH determinations were made with the glass electrode and the hydrogen electrode on solutions which were equimolecular with respect to piperazine dihydrochloride and the monohydrochloride (pK_1') and on solutions which were equimolecular with respect to the monohydrochloride and free piperazine (pK_2') (Table II).

Discussion

We have used the available data in Table II in making approximate calculations of the ionization exponents of piperazine at infinite dilution, since this has not been previously reported. Using the standard Debye-Hückel equation for moderately dilute solutions, we have found values for piperazine dihydrochloride for pK_1 of

TABLE I

Table for Preparation of Buffers at 25° C.

(1) 0.1591 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 0.1000 N sodium hydroxide as indicated below in column (1).
(2) 1.591 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
(3) 15.91 g. piperazine dihydrochloride diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
(4) 0.1321 g. glycylglycine diluted to 100 ml. with distilled water to which is added 0.1000 N sodium hydroxide.
(5) 0.1591 g. piperazine dihydrochloride plus 0.1321 g. glycylglycine diluted to 100 ml. with distilled water to which is added 1.000 N sodium hydroxide.
(6) 0.1591 g. piperazine dihydrochloride diluted to 100 ml. with filtered sea water (pH 8.0) to which is added 1.000 N sodium hydroxide.
(7) 0.1591 g. piperazine dihydrochloride plus 0.1321 g. glycylglycine diluted to 100 ml. with filtered sea water (pH 8.0) to which is added 1.000 N sodium hydroxide.

Buffer pH	ml. of NaOH to be added to above solutions						
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
4.4	0.66				0.007		
4.6	1.11	0.78			0.054		
4.8	1.71	1.18	7.0		0.120		
5.0	2.47	1.72	11.1		0.202		
5.2	3.38	2.48	17.4		0.302		
5.4	4.44	3.46	25.8		0.419	0.088	0.025
5.6	5.65	4.55	35.5		0.538	0.200	0.140
5.8	6.74	5.73	46.6		0.651	0.353	0.262
6.0	7.62	6.80	58.1		0.745	0.493	0.399
6.2	8.35	7.67	69.0		0.827	0.615	0.524
6.4	8.89	8.35	78.0		0.899	0.718	0.655
6.6	9.27	8.89	85.0		0.953	0.797	0.765
6.8		9.23	90.0		0.999	0.854	0.852
7.0			93.6	0.57	1.045	0.895	0.933
7.2				0.88	1.091	0.923	1.017
7.4				1.37	1.152	0.947	1.105
7.6				2.07	1.231	0.965	1.195
7.8				2.94	1.329	0.983	1.305
8.0				3.93	1.445	1.001	1.435
8.2				5.03	1.571	1.027	1.573
8.4				6.14	1.697	1.065	1.695
8.6	10.66			7.17	1.825	1.113	1.821
8.8	11.00	10.83	107.9	8.01	1.950	1.176	1.949
9.0	11.51	11.23	112.1	8.65	2.064	1.262	2.070
9.2	12.19	11.85	117.9	9.07	2.185	1.362	2.194
9.4	13.09	12.70	125.2	9.40	2.313	1.488	2.338
9.6	14.18	13.75	134.6		2.451	1.642	2.520
9.8	15.39	14.90	145.6		2.587	1.865	2.750
10.0	16.60	16.05	156.6		2.717		
10.2	17.68	17.09	166.6		2.832		
10.4	18.60	17.98	176.2		2.937		
10.6	19.35	18.74	184.4		3.042		
10.8		19.39	190.5		3.153		
11.0		19.94	195.2				

TABLE II

Relations Between pK' and Ionic Strength for Piperazine Dihydrochloride at 25° C.

Ionic strength	pK_1'	Ionic strength	pK_2'
0.0100	5.44	0.0174	9.74
0.0238	5.49	0.0519	9.78
0.100	5.56	0.174	9.82
0.238	5.68	0.571	9.87
1.00	5.79	0.800	9.88
1.67	5.86		

5.32 and pK_2 of 9.70. Bredig (1894) found a second ionization constant of 6.4×10^{-5} at 25° C., but failed to report a first ionization constant. Kolthoff (1925, 1925) reported a pK_1' of 4.05 and a pK_2' of 8.34 for piperazine at 15° C. Since the temperature he used is different from that used in the present experiment, the results are not comparable. A search of the literature reveals no other reports of the ionization exponents of piperazine.

On the other hand, because of interest in amphoteric electrolytes and in dipeptides, numerous studies of the K_a, K_b, pK_1', pK_2', pK_1, and pK_2 values of glycylglycine have been made (Euler, 1907; Dernby, 1916, 1917; Eckweiler et al., 1921; Harris, 1923; Levene et al., 1924; Täufel and Wagner, 1927; Branch and Miyamoto, 1930; Mitchell and Greenstein, 1930; Fromageot and Watremez, 1930; Stiasny and Scotti, 1930; Greenstein, 1933; Johnson and Peterson, 1935; Neuberger, 1937; Konikov, 1938; Carr and Shutt, 1939; Glasstone and Hammel, 1941; Smith and Smith, 1942). Included above are numerous titration curves for glycylgylcine in different media, but no actual tables for the preparation of buffers have been previously reported.

SUMMARY

The advantages of piperazine dihydrochloride and glycylglycine as buffers include the low toxicity, the lack of volatility, the solubility, the availability of the pure products, the convenience and accuracy of buffer preparation, and the lack of precipitation of calcium and magnesium salts from sea water below a pH of 9.9.

Table I indicates the preparation of solutions of known pH involving piperazine dihydrochloride, glycylglycine, and mixtures of these two in distilled water and in sea water at 25° C. Because the determinations were not made with the hydrogen electrode this table must be considered as being susceptible to small error, especially on the alkaline side of pH 9.

Table II shows the pK_1' and pK_2' values for piperazine dihydrochloride for several ionic strengths. Using the Debye-Hückel equation, the extrapolated pK values of piperazine at infinite dilution were found to be 5.32 for pK_1 and 9.70 for pK_2.

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CHROMATOPHOROTROPINS IN THE CENTRAL NERVOUS ORGANS OF THE CRAB, HEMIGRAPSUS OREGONENSIS

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INTRODUCTION

Early work on the humoral control of crustacean chromatophores has demonstrated that the sinus gland is the most important source of chromatophorotropic substances. Investigations leading to this conclusion are discussed in Brown's review (1944, pp. 130–134). Later work by Brown (1946) and Brown and Saigh (1946) has shown that most crustacean central nervous systems also possess at least two chromatophorotropic principles, one causing all portions of the body of Crago except the telson and uropods to become pale (Crago body-lightening hormone, CBLH), and a second (Crago-darkening hormone, CDH) darkening the telson and uropods, and in the absence of CBLH, the body also. CDH, however, was absent in the Brachyura studied.

This paper reports experiments undertaken for the purpose of determining whether or not chromatophorotropins are present in the central nervous system of the Pacific coast shore crab, *Hemigrapsus oregonensis*. Most attention was given to the optic ganglia, but some experiments were performed to test the brain and thoracic ganglia. I wish to thank Dr. R. I. Smith for his many helpful suggestions and criticisms.

MATERIALS AND METHODS

Only male crabs were used. Their eyestalks were ligated with number 80 cotton thread on successive days before injections were made. After the melanophores were completely punctate, the crabs were injected with Carcinides perfusion fluid (Pantin, 1934), and those whose melanophores responded at all were not used in subsequent experiments.

Organs from which extracts were made were rinsed in several changes of Carcinides perfusion fluid to remove any blood adhering to them and transferred to a roughened depression slide containing a drop or two taken from a measured quantity of perfusion fluid. Here they were torn apart, crushed, and triturated with fine forceps under a dissecting microscope, care being taken to ensure as complete extraction as possible. The extract was then transferred with an eyedropper to the measured quantity of perfusion fluid, and the depression slide was rinsed with this perfusion fluid several times. The extract was boiled for a few seconds, allowed to settle, and the supernatant fluid was used for injections. Sterile needles and syringes were used for all injections.

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To record the responses of the melanophores, an arbitrary index of four stages was used, from complete concentration of the pigment (stage 1) to complete dispersion (stage 4). The somewhat opaque and pigmented cuticle of *Hemigrapsus oregonensis,* especially in the larger crabs, obscures the chromatophores over much of the body, and observations were therefore made on the arthrodial membranes at the bases of the legs.

In preliminary experiments it was found that the melanophores of *H. oregonensis* responded to injections of sufficiently strong extracts of muscle and gill, as well as to weak egg albumin solutions (Fig. 2). Such preparations, particularly the latter, certainly do not contain chromatophorotropins, and the melanophore response in such cases probably is part of a rather generalized stimulation resulting from the introduction of foreign substances into the hemolymph. Since it was essential to eliminate such responses when testing extracts of central nervous organs for chromatophorotropins, this was done by making up the extracts from comparable volumes of tissue, small enough so that extracts of them would not contain sufficient protein or other unknown non-humoral material to affect the chromatophores. In this way it was intended to distinguish specific chromatophorotropic effects from the non-specific effects resulting from injection of large tissue masses. In order to make up such extracts it was necessary to know the amounts of tissue in the different organs extracted. Since the organs, especially the sinus glands, were too small to weigh on an analytical balance, measurements were made of their volumes using a procedure suggested by the method of Weil and Pantin (1931) for measuring volume changes in the turbellarian, *Gunda ulvae.* The organ was carefully dissected out, placed in the ruled area of a hemacytometer counting chamber and flattened under the cover glass. An enlarged (1 mm. = 2 in.) outline of the organ was drawn on a piece of paper containing a copy of the ruled area of the hemacytometer; this was traced onto a piece of medium weight drawing paper, cut out, and weighed. By comparing this weight with that of a similarly enlarged square millimeter (0.1 cu. mm.) the volume of the organ could be roughly determined. Table I gives the results of these measurements. All crabs used had a carapace width of 1.7 cm. The numbers are the weights of the paper cutouts in milligrams.

These measurements are admittedly crude, most of the error being due to dissection. They do, however, give some idea of the relative size of the organs involved. The dilution factors are selected values, based on the relative volumes by which extracts of the different organs were diluted to give approximately equal volumes of tissue in the same amounts of extract. For organs other than those listed in Table I (brain, leg nerve, etc.) the volume was measured by the preceding method, and the extract was diluted accordingly.

In preparing extracts, the size of the crab from which the organ was extracted was considered. It was assumed that the size of the sinus gland and other organs varies directly with the weight of the crab; thus, for example, the sinus gland from a crab 1.9 cm. wide (3.0 g.) would be twice the volume of that from a crab 1.5 cm. wide (1.5 g.).

The amount of extract injected was always 5 per cent of the body weight, assuming a specific gravity of 1.00 for the extract. To obviate weighing each crab, a large number of crabs were weighed and the weight plotted against the

TABLE I

Comparative size of sinus gland and optic ganglia

	Sinus gland	Medulla terminalis	Medulla interna	Medulla externa	Lamina ganglionaris	1 mm.[2] paper
	19	607	308	311	207	377
	14	767	284	293	282	364
	16	501	272	341	250	355
	18	579	249	250	198	365
	12	550	236	259	157	376
	14	350	210	279		374
av.	15.5	559	260	289	219	369
$\frac{\text{av.}}{3690}$ = mm.[3]	0.004	0.151	0.070	0.078	0.059	0.100
Relative volume	1	36.1	16.8	18.7	14.17	
Dilution factor	1	30	15	15	12	

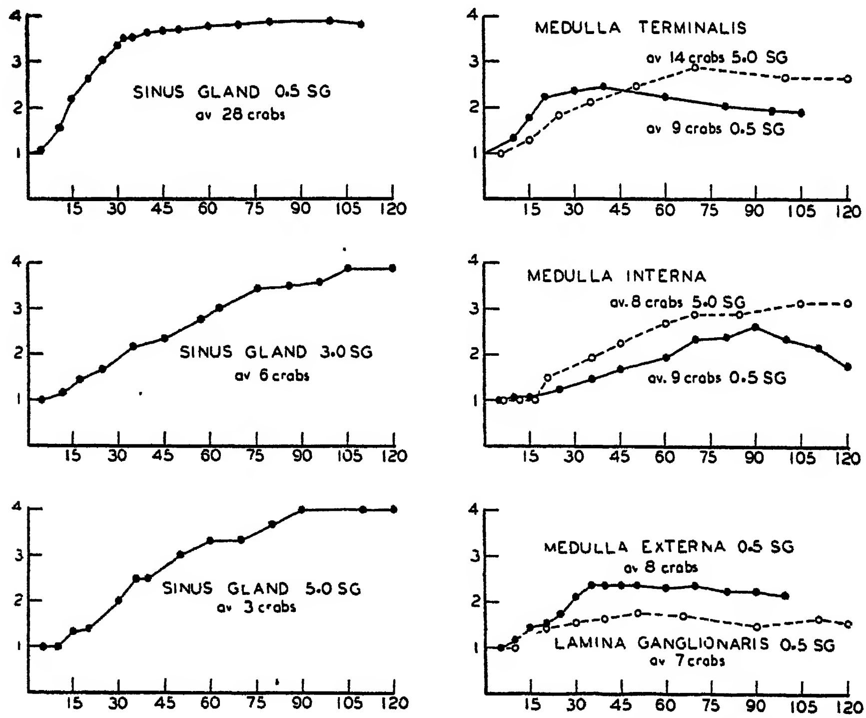

FIGURE 1. Responses of Hemigrapsus melanophores to injections of extracts of various organs. Abscissae: time (minutes) after injection. Ordinates: degree of dispersion of melanin (1 = complete concentration; 4 = complete dispersion).

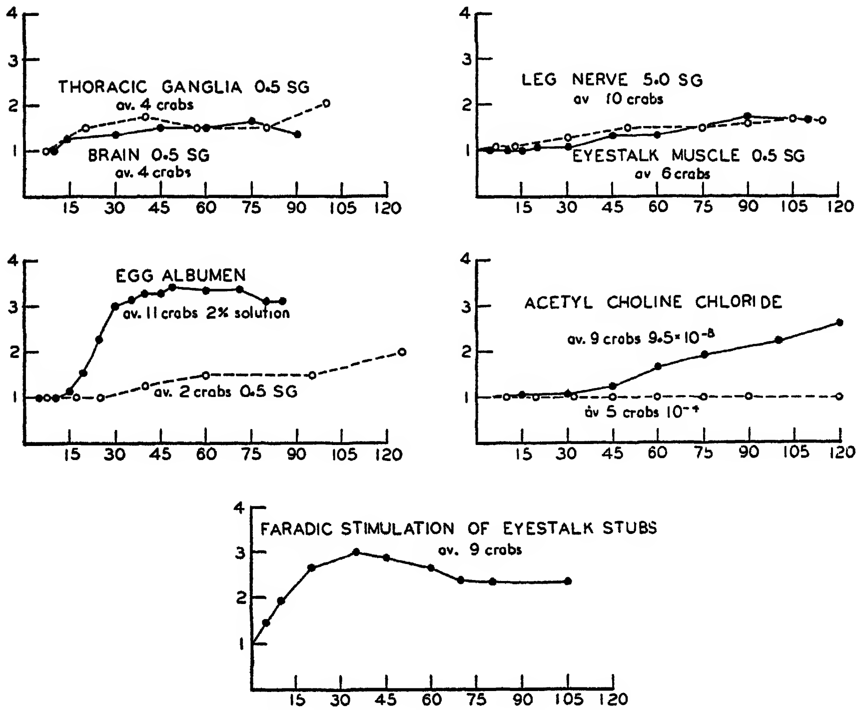

FIGURE 2. Responses of Hemigrapsus melanophores. Explanation same as for Figure 1.

carapace width. This made it possible simply to measure the carapace width and to inject the proper amount. Injections were made at the base of a walking leg.

The strength of each extract is expressed in terms of the volume of a sinus gland of the crab injected. Thus "0.5 SG" (cf. Fig. 1) indicates that a crab injected with 5 per cent of its body weight of this extract received a volume of tissue approximately equal to 0.5 times the volume of one of its own sinus glands.

EXPERIMENTS AND RESULTS

The results of the injections are shown in Table II and in the graphs of Figures 1 and 2. Responses are classified as "weak" when the melanin was not dispersed beyond stage 2, and "good" when stage 3 was reached. The average responses do not include those animals which failed to respond. The validity of averaging the arbitrary figures of the melanophore index is subject to criticism (Parker, 1948, pp. 14–15), and the variability of response, shown in Table II, must be considered when evaluating a response.

By far the most potent extracts were those of sinus glands. Other extracts from comparable volumes of tissue, while in some cases acting as rapidly as sinus gland extracts, did not produce the maximum and sustained responses which always followed injections of the latter. Moreover, the only extracts which always produced 100 per cent "good" responses were those of sinus glands. The more rapid

TABLE II

Summary of experiments

Organ extracted	Strength of extract (vol. of one of own sinus glands = 1)	No. of crabs injected	Responses		
			None	Weak	Good
Sinus gland	0.5 SG	28	0	0	28
Sinus gland	3.0 SG	6	0	0	6
Sinus gland	5.0 SG	3	0	0	3
Medulla terminalis	0.5 SG	11	2	4	5
Medulla terminalis	5.0 SG	15	1	5	9
Medulla interna	0.5 SG	13	4	5	4
Medulla interna	5.0 SG	8	0	1	7
Medulla externa	0.5 SG	8	0	5	3
Lamina ganglionaris	0.5 SG	17	10	5	2
Brain	0.5 SG	8	4	4	0
Thoracic ganglia	0.5 SG	12	8	3	1
Eyestalk muscle	0.5 SG	13	7	5	1
Leg nerve	5.0 SG	21	11	9	1
Egg albumin	0.5 SG	14	12	2	0
Egg albumin	2% sol'n	11	0	2	9
Acetylcholine	9.5×10^{-8}	26	17	5	4
Acetylcholine	10^{-4}	5	5	0	0
Faradic stimulation of eyestalk stubs		10	1	1	8

responses to the weaker (0.5 SG) extract are difficult to understand, but because of the small number of crabs injected with the stronger sinus gland extracts and the extent of individual variability in responsiveness, it is not possible to compare the responses adequately.

The responses to optic ganglia extracts with a concentration of 0.5 SG were in most cases weak, but these extracts probably did contain specific chromatophorotropins, since the responses to 0.5 SG egg albumin solutions were so slight, and the responses to eyestalk muscle extracts of the same concentration were also insignificant. However, the amounts of hormone in these optic ganglia extracts, especially those of lamina ganglionaris, appear to have been close to the threshold for the melanophores of *Hemigrapsus oregonensis*.

The responses to optic ganglia extracts of the concentration 5.0 SG were more definite, although they were much weaker than the responses to sinus gland extracts. It can be safely said, therefore, that the medulla terminalis and medulla interna (and probably the other optic ganglia) contain material that causes dispersion of the melanophores in *Hemigrapsus oregonensis*. The importance of this to the normal crab might be determined by removing the sinus glands without destroying the optic ganglia.

The possibility remained that the melanophores were responding to the acetylcholine present in the extracts. Welsh (1939) found large amounts of acetylcholine in leg nerves and ventral ganglia of Carcinides (= Carcinus), there being about five times as much in ganglia (about 10γ/g.) as in fibers (about 2γ/g.), while Smith (1939) found up to 20γ/g. in nerve fibers and up to 66γ/g. in

the ganglia of *Cambarus limosus*. It is improbable, however, that the chromatophorotropic effects of Hemigrapsus ganglia extracts are due to their acetylcholine content, since cholinesterase is probably also present in these extracts (Marnay and Nachmansohn, 1937). Moreover. Abramowitz and Abramowitz (1938) obtained slight responses of the chromatophores in only 20 per cent of the Uca they injected with acetylcholine. When injected with 5 per cent of their body weight of 9.5×10^{-5} acetylcholine chloride, each Hemigrapsus received the amount of acetylcholine that would have been present in tissue equal in volume to five times one of its own sinus glands, assuming 50γ/g. as the concentration of acetylcholine in this tissue. Seventeen out of twenty-six crabs thus injected failed to respond, and five crabs injected with the much stronger 10^{-4} acetylcholine also showed no response. This makes it fairly certain that the responses to optic ganglia extracts were not caused by the acetylcholine contained in them.

Responses to extracts of brain and thoracic ganglia of the concentration 0.5 SG were in most cases weak or absent, and they give little information as to whether or not chromatophorotropins are contained in these organs. A few injections of much stronger extracts of brain resulted in good responses, but these experiments were not well controlled. The good response to about ten seconds' faradic stimulation of one of the eyestalk stubs with a Harvard inductorium shows, however, that substances affecting the melanophores can be released in the eyestalkless Hemigrapsus, most probably from some part of the central nervous system. Deep probing of eyestalkless Hemigrapsus with a hypodermic needle at the base of the third or fourth leg also caused melanin dispersion in some cases. It is possible that the melanin dispersion following injections of muscle extracts and egg albumin solutions is an indirect response, caused by the release of chromatophorotropins from central nervous sources.

It is interesting to note that responses to leg nerve extracts were weak or absent, indicating that if a chromatophore hormone is present in nervous tissue it may be produced by or concentrated in the central nervous system rather than the peripheral nerves.

Discussion

It must be emphasized that these experiments do not compare the total amount of chromatophorotropic hormone available to the animal from one organ with that available from another organ, but indicate that while this hormone is most concentrated in the sinus gland it is not absent from certain parts of the central nervous system. It is entirely possible that in *Hemigrapsus oregonensis* as much or more hormone is present in central nervous system sources as in the sinus glands, although the present work does not provide quantitative information concerning this point. Although Brown (1940) found that 80 per cent of the chromatophorotropic material in the eyestalks of several species of shrimps and crabs was referable to the sinus gland, Smith (1948) has recently presented evidence that only about one-third of the retinal pigment activator in the eyestalks of *Hemigrapsus oregonensis* and two other species of grapsoid crabs resides in the sinus glands. It seems not unlikely that the distribution of the melanophore activator in the eyestalks of *Hemigrapsus oregonensis* is comparable.

The concentration of chromatophorotropins in the histologically specialized and

well innervated sinus gland may represent an adaptation for the storage and more especially the release of active substances in effective amounts and within short periods of time. Production of the active principles themselves might be by nervous tissues in general, or, as seems more likely, might be limited to more or less restricted regions of specialized cells within the central nervous system, including the sinus gland itself. Thus we could imagine that the chromatophorotropins in any given mass of nervous tissue are derived from a relatively few cells, each as specialized as sinus gland cells. These cells could be evenly distributed, resulting in a uniform distribution of hormone throughout the central nervous system, as Brown and Saigh (1946) found for CDH in the isopod, *Idothea baltica*. On the other hand, as in the case of CDH and CBLH in Crago, they could be restricted to a single organ (the tritocerebral commissure, Brown, 1946). The sinus gland, as Turner (1948, p. 561) points out, probably represents the highest evolutionary stage in the differentiation of endocrine tissue from the central nervous system in the Crustacea, and would therefore be expected to contain the highest concentrations of active materials. The experiments reported herein show this to be the case for chromatophorotropins in the sinus gland of *Hemigrapsus oregonensis*.

Summary

1. The melanophores of *Hemigrapsus oregonensis* become punctate after eyestalk removal.

2. Chromatophorotropins, which cause dispersion of the melanin when injected, are present in greatest concentration in the sinus gland, and are also present in the optic ganglia and possibly in the brain and thoracic mass of ganglia.

3. The melanin dispersion in response to electrical stimulation of the eyestalk stubs and to deep probing with a hypodermic needle indicates that some source of releasable chromatophorotropins exists other than the eyestalks.

4. The total amount of chromatophorotropins in the sinus gland is not necessarily greater than in any of the central nervous organs. The specialized structure and the innervation of the sinus gland suggests that its importance lies in its ability to store and rapidly release effective amounts of chromatophorotropins.

5. Injection of sufficient amounts of certain substances, including muscle and gill extracts and egg albumin solution, also induces melanin dispersion in eyestalkless *Hemigrapsus oregonensis*. It is suggested that these substances do not contain chromatophorotropic hormones, but the response to them is the result of a more general stimulation causing the release of chromatophorotropins from central nervous sources.

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SOME EFFECTS OF CENTRIFUGING UPON PROTOPLASMIC STREAMING IN ELODEA [1]

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As pointed out by Ewart (1903), protoplasmic streaming was probably observed before the existence of protoplasm as such was recognized. Notwithstanding the many studies that have been made upon this interesting and complicated biological phenomenon, certain of the basic problems such as motive force, function and mechanism of flow remain largely unsolved. This is not surprising, because to understand protoplasmic streaming requires a rather complete knowledge not only of protoplasm itself, but also of the physics and chemistry of streaming as well. In fact, certain theories of protoplasmic structure are inadequate because they fail to account for a suitable structural mechanism to allow for protoplasmic streaming.

Of the extensive literature dealing with protoplasmic streaming, few papers have been published which are concerned directly with the effects of centrifugal force on this process as such, although numerous studies have been made upon protoplasmic viscosity by aid of the centrifuge. Accordingly, it seems desirable to record here the results of some studies made upon protoplasmic streaming in the leaf cells of Elodea by use of the ultracentrifuge.

Material and Methods

Leaves of *Elodea canadensis* were removed from a region one to two inches back of the tip of an actively growing stem. They were placed in a dish where about one-fourth inch of the tip was cut off in order that the piece be of suitable size to fit in the cell of the air-driven rotor. The tips were then placed directly in a water mount where they were observed under the high dry and oil immersion lenses. Here observations were made upon the frequency and direction of streaming in the cells of a selected area near the tip of the leaf. The piece was then removed to the rotor of the ultracentrifuge with the long axis parallel to the centrifugal force. It was then centrifuged at forces varying from 135,000 to 350,000 times gravity for intervals varying from five minutes to four hours. After centrifuging, the piece was again removed to a slide and the region formerly studied selected for observation.

Some of the pieces, after having the cells stratified in one direction, were reversed in the centrifuge so that stratification in the opposite direction occurred. Certain of the pieces were killed immediately upon removal from the ultracentrifuge by immersion for a short interval in boiling water.

The minimum time required to stop the centrifuge, remove the piece to a slide and find the area formerly studied was about three to four minutes.

[1] Aided by grant from the National Institutes of Health, administered by J. H. Bodine.

Observations

As is well known, the long axis of the cells of Elodea is arranged parallel to the long axis of the leaf. Near the mid-rib of the leaf the cells are usually longer and narrower and are more likely to be found undergoing streaming. Usually, if the leaf has been taken from near the tip of an actively growing stem, a few minutes' stand on the stage of the microscope, exposed to light and a slightly elevated temperature, is sufficient to initiate active rotational streaming in a high percentage of the cells. The streaming in Elodea has been referred to as rotational (Seifriz, 1943) because the protoplasm is chiefly confined to a peripheral layer between the sides of the cell and the central vacuole. In Plate I, Figure 1, is illustrated a control cell in active streaming. The chloroplasts at the periphery are blurred because of their movement within the interval required to take the photograph.

Under normal conditions, according to Pfeffer (1906), the flow is in one direction only within the cells, clockwise or counter clockwise. In addition, both Pfeffer (1906) and Ewart (1903) maintain that the flow is in opposite directions on the two sides of the dividing walls between each pair of contiguous cells. We have not been able to confirm this statement in the Elodea studied here. Counts of a hundred pair of actively streaming adjacent cells revealed that in only about 70 per cent of them was the direction of flow opposite on the two sides of adjacent cell walls. The reason for this discrepancy is not clear, but it should be recalled that Berthold (1866) reported inconsistency in the direction of streaming between adjacent cells of Elodea. However, why, in the majority of adjacent cells, the direction of streaming on the two sides of the adjacent walls should be opposite, is unknown.

Figure 2 shows a low power view of a number of cells that were centrifuged at approximately 135,000 times gravity for five minutes. It will be observed that the chloroplasts are packed at the centrifugal pole. If the rotor is allowed to accelerate rapidly, the chloroplasts, but not the cytoplasm, are probably displaced within ten seconds. In other words, the movement of the chloroplasts takes place very rapidly through the protoplasm of the cell. It is of interest that the protoplasm is not killed by this rapid displacement of the chloroplasts through it. In fact, stratified cells (Fig. 4) may be reversed in the centrifuge and the chloroplasts thrown to the opposite end (Fig. 5). This process may be repeated several times without killing the cell. However, Northen and Northen (1938) have found that repeated centrifugation and displacement of the chloroplasts through the cells of Spirogyra produces a marked lowering of the viscosity.

High centrifugal force, 350,000 times gravity (Fig. 3), causes rapid displacement of the cellular materials. From the centrifugal to the centripetal pole the elements are stratified as follows: (1) chloroplasts, cytoplasm and nucleus; (2) vacuole. It will be noted that a sharp boundary exists between the centrifugal end of the vacuole and the cytoplasm, although such is not always the case for the chloroplasts, cytoplasm and nucleus. Apparently the vacuole is displaced centripetally and is forced in contact with the cell wall. No cytoplasm can be observed remaining along the sides of the cell. What happens to the plasma membrane if such be a permanent structure in Elodea, is not known. No evidence of

PLATE I

organized streaming of the protoplasm is apparent in such cells, although Brownian movement may be evident.

Figures 6, 7, 8 and 9 are a series of photographs showing stratification and partial recovery of three cells centrifuged for thirty minutes at 350,000 times gravity. A complete stratification of the cellular contents occurs here (Fig. 6) as was also noted in Figure 3. Immediate examination upon removal from the ultracentrifuge revealed the cytoplasm in Brownian movement. All indications of any type of organized streaming or even churning movements are absent. However, within thirty minutes after centrifuging, the Brownian movement of the cytoplasm pushes the chloroplasts back beyond the edge of the vacuole so that it is no longer obvious (Fig. 7). A gradual migration of the cytoplasm toward the centripetal end, followed by a movement of some of the chloroplasts along both sides of the cells, occurs. There is in these cells no organized streaming of cytoplasm at this time. Within sixty minutes after centrifuging (Fig. 8), the cytoplasm has become continuous around the sides of the cells and has started to flow in an organized fashion. However, the rate of flow is very slow when compared to that of the normal cells. Ninety minutes after centrifuging (Fig. 9), the chloroplasts in the upper two cells are still largely bunched at the centrifugal end although some isolated chloroplasts are observed being carried around the cell in the stream. In the lower right hand cell the bunched chloroplasts are rotating around the cell as a mass. However, the movement is not continuous but by jerks, due to the forces encountered in trying to move a large mass through a relatively small stream. In some cells treated in this manner, complete recovery with normal streaming and normal distribution of chloroplasts occurs. In others, the cells are killed. In still others, as in Figures 6, 7, 8 and 9, injury occurs from which the cells partially, but not completely, recover within a six-hour interval. Less than one per cent of the cells have their walls ruptured and their contents missing (Fig. 15).

A cell centrifuged at 135,000 times gravity for five minutes is illustrated in Figures 10, 11, 12 and 13. Here the chloroplasts are not as tightly packed as they are in the cells centrifuged at higher forces. Figure 10 shows the cell about four minutes after removal from the centrifuge. In addition to the chloroplasts the nucleus may be clearly observed. Rapid Brownian and churning movements of the protoplasm are evident at the centrifugal end of the cell, and the massed chloro-

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PLATE I

All figures in Plates I and II (except Fig. 15) are of living unstained cells of Elodea. In all centrifuged cells the force was directed at various angles toward the bottom of the plate except when otherwise indicated.

FIGURE 1. Control cell actively streaming.

FIGURE 2. Low power view of the general effects of centrifugal force on the position of the chloroplasts. Centrifuged at approximately 135,000 times gravity for five minutes.

FIGURE 3. High power view of cells showing stratification effects. Centrifuged at 350,000 times gravity for thirty minutes.

FIGURES 4 AND 5. Same cell centrifuged first in one direction and then in the opposite direction (at 135,000 times gravity for five minutes in each case). Centrifugal force in Figure 5 is toward the top of the plate.

FIGURE 6. Group of cells photographed about six minutes after being centrifuged at 350,000 times gravity for thirty minutes.

FIGURE 7. Same cells thirty minutes later.

PLATE II

plasts are being pushed centripetally. Here the reestablishment of flow is up the right side of the cell. The chloroplasts gradually migrate in the stream along the right side of the cell (Figs. 11, 12) and eventually a complete separation of them occurs and recovery is effected (Fig. 13). In this cell the nucleus was not observed to be actively carried about in the streaming cytoplasm. However, they often do rotate in cells so treated. Figure 14 is a cell treated as in Figure 10. Here the redistribution of the chloroplasts is along both sides of the cell instead of one side only. In other words, organized streaming was delayed in this cell for a longer time than it was in the cell in Figure 10, and recovery was eventually established.

In addition to the methods of recovery already described for centrifuged cells, other conditions may occur. In some cells the packed chloroplasts at the centrifugal end may be forced back as a mass to a position near the middle or even to the centripetal end (Figs. 16, 17). This seems to occur as a result of the clumped chloroplasts partially blocking the movements of the redistributing cytoplasm. Consequently, they are simply forced ahead of it. The nucleus, too, may be carried along with the massed chloroplasts. When the cytoplasm breaks around the chloroplasts or develops new streams across the cell, the clumped chloroplasts break away into the stream, usually in small groups at first, but eventually they become isolated. It may take thirty minutes or more for normal distribution of the chloroplasts to become established.

Observations on the direction of streaming in one hundred cells that were subsequently centrifuged at 135,000 times gravity for five minutes, showed that approximately 7 per cent of them had undergone a reversal in direction of flow.

Clumped chloroplasts such as those shown in Figures 16 and 17 sometimes, but not always, cause formation of protoplasmic strands which cross between the wall and vacuole in almost any direction, resulting in many diverse and irregular patterns of flow. Two or more protoplasmic strands may join on the upper surface of the vacuole, giving rise to a churning motion of the protoplasm at the point of juncture. Flow in a single strand may be in opposite directions. When this condition occurs, the velocity of flow is much slower at the point of contact of the two opposite flowing protoplasmic streams. The small granules moving in

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PLATE II

FIGURE 8 Same cells sixty minutes later.

FIGURE 9. Same cells ninety minutes later.

FIGURES 10, 11, 12 AND 13 Series of photographs showing recovery from centrifuging at 135,000 times gravity for five minutes.

FIGURE 14. Cell in process of recovery from centrifuging at 135,000 times gravity for five minutes.

FIGURE 15. Cell showing broken wall and chloroplasts missing Centrifuged at 350,000 times gravity for thirty minutes. Such conditions are rare.

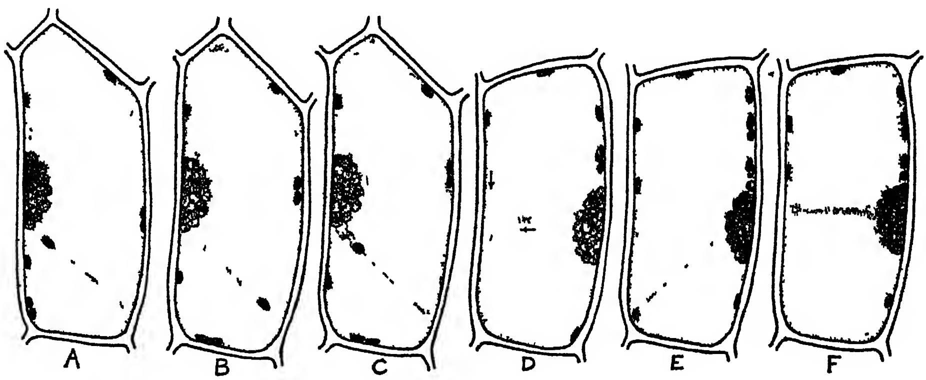
FIGURES 16 AND 17. Cells centrifuged at 135,000 times gravity for five minutes Photograph taken ten minutes later. The chloroplasts are being forced back from the centrifugal end by Brownian movement of the cytoplasm (Centrifugal force directed to left in figures)

FIGURES 18, 19 AND 20. Starch grains in various stages of displacement within chloroplasts The starch grains are on end of chloroplasts originally directed centrifugally. Brownian movement has caused the chloroplasts to lose the position taken up in the centrifugal field.

FIGURE 21. Starch grain that has been freed from the chloroplast presumably by the centrifugal force

opposite directions at the point of contact of the two streams may be observed to collide and bounce around each other. Streaming in opposite directions within a single strand is usually a temporary condition. Direction of flow in the strands has also been observed to be in one direction for a time and subsequently reversed. Reversal of flow, however, is usually of short duration and appears to be caused by the difference in flow pressure produced by the main channels of the cytoplasm along the two opposite sides of the cells in which the strands are usually directly or indirectly connected. The difference in flow pressure of the two opposite sides of the cells is often due to the massed chloroplasts blocking the normal channels of flow. Hence, strands are developed at the points of least resistance to the protoplasmic flow.

In some of the thin protoplasmic strands which extend across the cell, a chloroplast may become included within its streaming cytoplasm (Text Fig. 1, A). The chloroplast, being greater in diameter than the stream, moves relatively slowly along its channel (Text Fig. 1, B). In a few such cases we have observed that



Text Figure 1

Figs. A, B and C. Centrifuged cell partially recovered showing movement of chloroplast by means of contraction within small strand. At A the chloroplast is flowing slowly toward the lower right side of cell. At B it has almost reached the opposite end of strand. However, suddenly a rapid backward movement of the chloroplast occurs as shown at C.

Figs. D, E and F. These figures illustrate contraction of a cytoplasmic strand. Figure D shows position of strands. At E strand has moved with current down along side of cell. Figure F shows return of strand to near former position.

a quick return of the chloroplast from a position indicated in Text Figure 1, B to that of Text Figure 1, C occurs. Here the reversed movement of the chloroplast seems to be due to a contraction of the strand, because its movement is much too fast to be accounted for as a simple reversal of flow of the cytoplasm within the strand.

Other protoplasmic strands extending across the cell from the upward channel of flow of one side of the cell to the downward channel of flow of the other side sometimes change position rapidly (Text Fig. 1, D, E and F). For example, the end of the strand in connection with the downward channel of flow may move with the current to a position indicated in Text Figure 1, E. When this occurs

the strand appears to be stretched and its diameter diminished. However, suddenly such a strand may "snap back" very quickly to a point near its former position (Text Fig. 1, F). This change seems to be due to a property of the protoplasm of the strand as a whole. The contraction described above may be repeated several times. We have also observed that "balls" of protoplasm considerably larger than the diameter of the strand may flow along its channel. Such occurrences are not rare and have been described by others in normal protoplasmic streaming.

Figures 18, 19, 20 and 21 are centrifuged cells with chloroplasts showing starch grains. The starch inclusions appear heavier than the chloroplasts and are displaced centrifugally. Figure 18 illustrates a chloroplast with the starch grain displaced so that it appears to be partially extruded. The positions taken up by smaller starch grains are indicated in Figure 19. The upper chloroplast in Figure 20 shows not only a partially displaced starch inclusion, but evidence of a partial stratification of the other materials as well. However, this is rare, for in the majority of the cells little, if any, stratification can be detected within the unstained chloroplast. This indicates either that the chloroplast as a whole is very dense, or that its contents vary only slightly in relative specific gravity.

In Figure 21 the lower inclusion is an isolated starch grain which has probably been pulled away from the chloroplast by the centrifugal force. It is difficult to say anything definite concerning the nature of the surface "membrane" of the chloroplast other than that considerable resistance is met at the surface in the displacement of the starch grains through it. In other words, the presence of some form of limiting "membrane" is indicated, although such a structure could not be seen in the centrifuged unstained chloroplast.

Discussion

It is unnecessary here to review the extensive literature dealing with protoplasmic streaming, since excellent reviews on this subject have been published by Ewart (1903) and more recently by Seifriz (1943).

Protoplasmic streaming is known to be affected by many agents such as temperature, visible light, ultraviolet light, salts, acids, alkalies, oxygen, organic substances, anesthetic agents, x-rays, radium. electricity, hydrostatic pressure, mechanical manipulation and supersonic waves (see Seifriz, 1943, for references). In this paper it has been demonstrated that it is also affected to varying degrees by ultracentrifugal force. However, both Andrews (1915) and Vexler (1935) report that centrifuging with the usual laboratory type centrifuge produces little effect upon protoplasmic streaming. In fact Vexler (1935) found that centrifuging stimulated streaming in Myxomycetes.

Marsland (1939) has suggested that in Elodea the streaming is motivated by sol-gel reactions and consequently is a phenomenon fundamentally related to ameboid movement. The evidence for this is that with increasing hydrostatic pressure, the rate of protoplasmic streaming in Elodea is diminished. Complete obliteration of streaming with decreasing viscosity of the cytoplasm occurs at between 400 to 500 atmospheres. This reaction is reversible. To apply the theory of sol-gel reversibility of protoplasm as the chief mechanism of streaming in a cell like Elodea is not easy, for according to Pfeffer (1906) only a very thin ectoplasmic membrane

exists in contact with the cell wall in actively streaming cells. Furthermore, according to Ewart (1903), the adjacent surfaces of both the protoplasm and vacuole flow. In addition, to account for rotational streaming by a sol-gel mechanism requires several assumptions. Because of this, Seifriz (1943) suggests an alternate view, namely, that "streaming is occasioned by a contractile force which need not involve a viscosity change or a sol-gel transformation."

If the sol-gel mechanism is the main source of the motive force in protoplasmic streaming in Elodea, as there is certainly good evidence that it is for protoplasmic streaming in certain other cells (Mast, 1931; Lewis, 1942), the inhibition of streaming produced by centrifuging may be due to a lowering of the viscosity resulting in a disruption of the sol-gel mechanism.

Moore (1935) found that centrifuging at 75,000 times gravity for five minutes deforms and retards proliferation (and I presume streaming) for fifteen hours in plasmodium. He interprets this result as due to a separation of a heavy and light component of the cytoplasm. When allowed to stand for sufficient time, these heavy and light components of the cytoplasm return to their normal spatial relationships, and proliferation and streaming are reestablished.

High centrifugal force has been demonstrated to displace most of the visible cellular materials (Beams and King, 1939; Beams, 1943) and certain ultramicroscopic structures in the liver cell (Claude, 1943), as well as various types of protein molecules in non-living colloidal solutions (Svedberg, 1934). Yet, convincing evidence has not been obtained that a disruption of the vital ultramicroscopic organization of the protoplasm of Ascaris eggs occurs even at forces on the order of 900,000 times gravity for thirty minutes (Beams and King, 1937; Beams, 1943). This indicates that the intermolecular forces contributing to the vital structural protoplasmic framework are sufficiently great to resist disruption by high centrifugal force.

However, whether or not the inhibition of protoplasmic streaming in Elodea reported here may be explained on the same basis as that given by Moore (1935) for plasmodium is unknown.

In spite of the fact that all the visible cellular materials, including the cytoplasm, may be displaced to one end of the cell, recovery in the majority of cells does not result in a reversal of the direction of streaming. No experimental procedure has as yet been developed to produce consistent reversal in streaming of Elodea cells. However, Ewart (1903) states that change in direction of streaming can sometimes be observed in cells of Elodea after the application of stimuli sufficiently great to produce death in some cells and temporary stoppage of streaming in others. Seifriz (1943) has observed in treated Elodea that the chloroplasts flow as a belt around the "waist" of the cell. It is difficult to understand where the factors for polarity of streaming reside within the cell. All of the cytoplasm, including the peripheral membrane, seems to be displaced, at least insofar as could be detected with the microscope.

Both elasticity and contractility have been observed in streaming cytoplasmic strands. In thin strands the thickness of the cortical layers must be very small. Hence, the contractile properties of the strand probably include the whole of its protoplasmic structure.

The chloroplasts seem to be of a highly viscid consistency. The starch granules were the only elements within them that could be consistently displaced by high

centrifugal force. This is in agreement with the observations made with the micro-dissection apparatus, to the effect that chloroplasts appear to be composed of an "elastic jelly of doughy consistency" (Scarth, 1927). Large starch grains often appear in normal storage tissue attached to the surface of a chloroplast (Zirkle, 1926). However, there seems little doubt that the displacement of the starch grains reported here is due to centrifugal force.

Conclusions

High centrifugal force produces a rapid stratification of the visible cellular materials in Elodea. From the centrifugal to the centripetal end, the materials are stratified as follows: (1) chloroplasts, cytoplasm and nucleus; (2) vacuole. In cells examined immediately after centrifuging at high forces, evidence of organized streaming is usually absent, but Brownian movement is often apparent. Most of the cells survive exposure to 350,000 times gravity for thirty minutes.

The inhibition of streaming may be due to a lowering of the viscosity as a result of the rapid displacement of the chloroplasts through it.

Recovery from centrifuging usually occurs in the following order: (1) Brownian movement at the centrifugal end aids in a redistribution of the granular cytoplasm along one or both sides of the cells; (2) initial streaming movements are usually of an unorganized type; and (3) organized streaming is slowly established which results in a slow irregular rotation of the large massed chloroplasts. Eventually the bunched chloroplasts separate, and the normal velocity of streaming, as well as the normal distribution of chloroplasts, is established.

In only about 7 per cent of the cells could the direction of streaming be reversed by centrifuging.

Evidence of elasticity and contractility within thin strands of protoplasm has been observed.

Displacement of starch grains within chloroplasts has been observed.

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THE PRESENCE OF THE TRICARBOXYLIC ACID CYCLE IN THE CILIATE COLPIDIUM CAMPYLUM [1]

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Colpidium campylum is a ciliate which can be easily cultured bacteria-free in a liquid medium. It has been demonstrated that when cultured in proteose-peptone from which the lipids have been extracted, the organism is capable of synthesizing large amounts of fatty acids (Wilber and Seaman, 1948).

Since the tricarboxylic acid cycle is a link between protein and carbohydrate metabolism, it seemed desirable to make a study of this cycle as the first step toward the elucidation of the pathway for fatty acid synthesis from protein in this organism.

While there have been many investigations of this cycle in vertebrate tissue and in bacteria, there has been little done with protozoa. Van Niel, Thomas, Ruben and Kamen (1942) found that the ciliate *Tetrahymena geleii* assimilates carbon dioxide in the anaerobic formation of succinate during the fermentation of glucose. Baker and Baumburger (1941) found cytochrome c, b, and a_1 to be present in this same organism with indications of the presence of cytochrome a_2. Hutchens, Jandorf and Hastings (1941) ascertained the DPN content of the flagellate *Chilomonas paramecium.* Hutchens (1940) also identified the presence of cytochrome c in Chilomonas. Laurie (1935) demonstrated the presence of succinic dehydrogenase in the ciliate *Glaucoma pyriformis.*

Materials and Methods

Colpidia were grown in sterile, pure cultures in 150 cc. Erlenmeyer flasks containing 50 cc. of 3 per cent Difco proteose-peptone solution from which the carbohydrate had been precipitated with copper sulfate (Peters and Van Slyke, 1931) and the lipids extracted with hot alcohol (Bloor, 1943). The organisms used were obtained from cultures maintained in the Biological Laboratory, Fordham University and are the same strain as was used in a previous investigation (Wilber and Seaman, 1948). For use in this investigation, new cultures were inoculated with 1 cc. of organisms from a three-day culture and allowed to grow for two days at a temperature of $22 \pm 2°$ C. At this time the cultures were at the mid-point of the logarithmic phase of growth (population about 40,000 colpidia per cc.).

The organisms for use were concentrated by centrifugation and aliquots with-

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[2] The author wishes to express his sincere gratitude to Dr. Charles G. Wilber for invaluable aid and encouragement.

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drawn for ascertaining the dry weights of the cells (Ormsbee, 1942). The remaining cells were washed three times with Hahnert's solution (Hahnert, 1932), to which was added magnesium sulfate to make a final concentration of 0.02 M (final pH adjusted to 5.6). The cells were then starved for twelve hours before use. At the end of this period the organisms were again concentrated, resuspended in the modified Hahnert's solution and 2 cc. portions (containing approximately 10 mg. dry weight of cells) transferred into standard Warburg vessels.

Oxygen uptake was measured by the conventional Warburg direct method. In all cases the total volume of each vessel was 3.5 cc. Vessels were shaken at a rate of 120 cycles per minute through an arc of 5 cm.

Sodium pyruvate was prepared by the method of Robertson (1942); oxaloacetic acid by the method of Krampitz and Werkman (1941). All other substrates were obtained commercially. Concentrations of substrates are given as final concentration.

Pyruvic and α-ketoglutaric acids were estimated according to the method of Friedmann and Haugen (1943); succinic acid according to Krebs (1937); oxaloacetate according to Edson (1935); fumaric acid according to Krebs, Smyth and Evans (1940).

Results

Pyruvate is rapidly metabolized by Colpidium. When 0.02 M pyruvate is added to cells respiring in modified Hahnert's solution there is an immediate

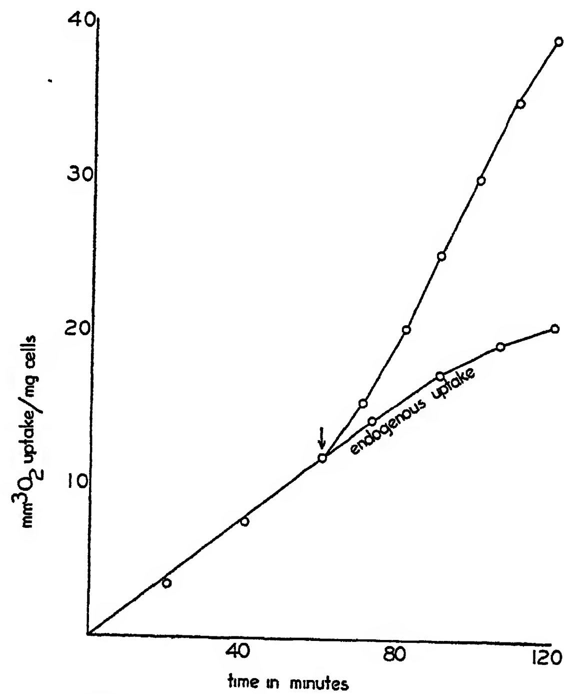

FIGURE 1. Effect of pyruvate on oxygen uptake in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. At arrow, 0.02 M pyruvate added.

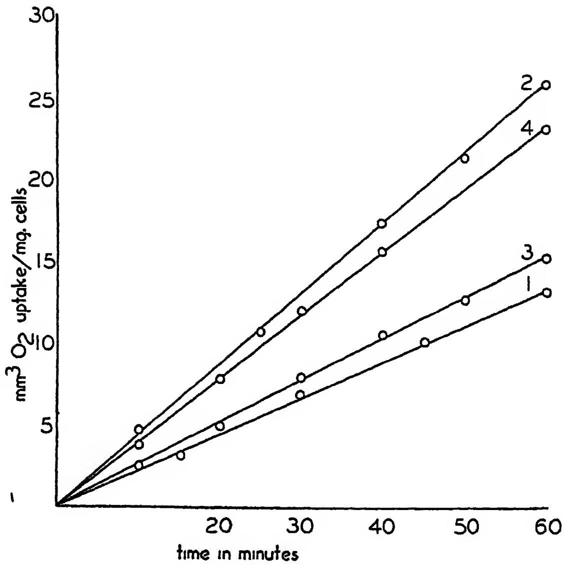

FIGURE 2. Effect of malonate and fumarate on oxygen uptake in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. Curve 1, no added substrate; curve 2, 0.02 M pyruvate; curve 3, 0.02 M pyruvate + 0.02 M malonate; curve 4, 0.02 M pyruvate + 0.02 M malonate + 0.001 M fumarate.

increase in the rate of oxygen uptake (Fig. 1). There is a utilization of 0.081 mg. of pyruvate per mg. dry weight of cells per hour (Table II).

If the tricarboxylic acid cycle plays a role in the metabolism of Colpidium, the oxygen uptake in the presence of pyruvate should be inhibited by malonate. This inhibition should be released upon the addition of fumarate. Figure 2 shows that 0.02 M pyruvate increases the Q_{O_2} from the endogenous value of 13.2 to 26.3, an increase of 99 per cent. In the presence of pyruvate and 0.02 M malonate the Q_{O_2} is 15.4, 83 per cent inhibition of the pyruvate effect. The Q_{O_2} is restored to a value of 23.6 by the addition of 0.001 M fumarate, an 89 per cent recovery of the malonate inhibition.

The effect of other acids of the tricarboxylic acid cycle on oxygen uptake is shown in Table I. Succinate results in an increased Q_{O_2} of 105 per cent; α-ketoglutarate 102 per cent; fumarate 90 per cent; malate 97 per cent; and oxaloacetate, an increase of 85 per cent.

The quantities of metabolites recovered from various substrates are shown in Table II and III. Fumarate and α-ketoglutarate are recovered in approximately equal amounts when pyruvate is the substrate. The addition of fumarate to pyruvate increases the recovery of α-ketoglutarate by 142 per cent. Fumarate and pyruvate are recovered in a ratio of approximately 1 to 4 when oxaloacetate is utilized as a substrate.

Table III shows that as a result of the fumarate release of malonate inhibition, there is an added utilization of 0.051 mg. pyruvate per mg. dry weight of cells per hour, and an added recovery of succinate amounting to 0.013 mg. per mg. dry weight of cells per hour.

TABLE I

Effect of acids of the tricarboxylic acid cycle on oxygen uptake in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. Concentration of all substrates except fumarate, 0.02 M; fumarate, 0.001 M.

Q_{O_2}	Substrate
13.2	—
27.1	succinate
26.8	α-ketoglutarate
25.1	fumarate
26.1	malate
24.5	oxaloacetate

TABLE II

Utilization of substrates and recovery of intermediate metabolites in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. $Q_{substrate}$ is mg. substrate utilized (−) or mg. metabolite formed (recovered) (+) per mg. dry weight of cells per hour. Pyruvate, 0.02 M; oxaloacetate, 0.002 M; fumarate, 0.001 M.

$Q_{substrate}$	Substrate added		
	pyruvate	pyruvate + fumarate	oxaloacetate
$(+)_{pyruvate}$	—	—	0.044
$(-)_{pyruvate}$	0.081	0.065	—
$(+)_{ketoglutarate}$	0.012	0.029	—
$(-)_{oxaloacetate}$	—	—	0.121
$(+)_{fumarate}$	0.016	—	0.014

TABLE III

Formation of succinate in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. Malonate, 0.02 M; pyruvate, 0.02 M; fumarate, 0.001 M.

$Q_{substrate}$	Substrate added (in addition to malonate)	
	pyruvate	pyruvate + fumarate
$(-)_{pyruvate}$	0.016	0.067
$(+)_{succinate}$	0.003	0.016

TABLE IV

Effect of succinate, α-ketoglutarate, and citrate in releasing malonate inhibition in Colpidium. Modified Hahnert's solution, pH 5.6. Gas phase, O_2. Temperature, 25.5° C. Malonate, pyruvate, α-ketogluterate, succinate, 0.02 M; citrate, 0.008 M; fumarate, 0.001 M.

Substrate (in addition to pyruvate which was present in all vessels)	Q_{O_2}
— — — —	26.3
malonate	15.4
citrate	26.1
citrate + malonate	13.7
citrate + malonate + fumarate	22.8
α-ketoglutarate + malonate	28.4
succinate + malonate	27.6

Added citrate in final concentrations ranging from 0.002 M to 0.01 M has no effect on the oxygen uptake. Table IV shows the ability of citrate in releasing malonate inhibition as compared to the ability of succinate and α-ketoglutarate to release the inhibition. Succinate and α-ketoglutarate release the malonate inhibition to approximately the same extent as does fumarate (compare Fig. 2), whereas citrate does not release the inhibition.

Discussion

It would be desirable to compare the Q_{O_2} values obtained for Colpidium in this investigation with values obtained for other protozoa. However, it is impossible to make such a comparison, since it was found (Ormsbee, 1942) that the Q_{O_2} of the same species of Tetrahymena varies from 6.2 to 77.7 depending upon the age of the culture, the length of the starvation period before oxygen uptake is measured, and the composition of the suspending medium. Other factors affecting Q_{O_2} values in protozoa are the rate of shaking of the manometer vessels (Hall, 1938) and the concentration of cells used (Pace and Lyman, 1947). Hutchens (1941) found that in *Chilomonas paramecium* the oxygen uptake per hour per 10,000 cells varies with different strains, even though both strains are studied under identical conditions.

Since added citrate does not increase oxygen uptake or release malonate inhibition, and since fumarate, succinate, and α-ketoglutarate do cause increased oxygen uptake and do release malonate inhibition, it must be concluded (Stare, Lipton, and Goldinger, 1941) that citrate does not occupy a major position in the tricarboxylic acid cycle as it occurs in Colpidium.

It appears from the data of Von Dach (1942) that the tricarboxylic acid cycle is not present in the colorless flagellate, Astasia. In this organism, succinate, fumarate and malonate have no significant effect on the oxygen uptake. In *Paramecium caudatum,* succinate increases oxygen uptake by only 8 per cent (Leichsenring, 1925).

Elliott (1935) found that pyruvic acid (0.5%) inhibits growth in *Colpidium campylum* and in *C. striatum.* On the other hand, Bond (1933) found that pyruvic acid stimulated growth in *C. campylum.* However, he found that succinate (1.0%) and malate (1.0%) inhibit growth. These findings are unusual if, as has been demonstrated in this paper, these compounds are metabolites. It must be noted that the concentrations used by these authors were very much higher than those used in the present investigation. It is well known that normally occurring metabolites in high concentrations may cause inhibition of metabolic functions, as measured by oxygen uptake. It would be desirable to ascertain the effects of acids of the tricarboxylic acid cycle on the growth of Colpidium when used in concentrations which are known to be physiologically active (0.001–0.02 M). Such an investigation is now in progress.

Summary

1. Evidence is presented for the presence of the tricarboxylic acid cycle in the metabolisms of the ciliate *Colpidium campylum.*

2. Apparently citrate does not occupy a major position in the tricarboxylic acid cycle as it occurs in Colpidium.

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GAMETOGENESIS IN THE OYSTER UNDER CONDITIONS OF DEPRESSED SALINITY

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The American oyster, *Ostrea virginica* Gmelin, flourishes naturally in brackish waters ranging in salinity from 16 to 27 parts per thousand. But the salt toleration of the animal is such that it can survive in waters having a much broader range of salt content. In many localities, commercial production of oysters is maintained where seasonal floods may expose the bars to entirely fresh water for short periods of time. Some of the more important seed-producing areas on the Atlantic Coast consistently have a salt content of less than 15 $^0/_{00}$. Consequently the effects of lowered salinity on oyster physiology and reproductive ability have long been of interest. The opportunity presented itself in 1946 to examine the gonads of oysters living under unusually great variations in salt content. Extensive flood waters from the Susquehanna River watershed into the upper reaches of Chesapeake Bay during the summer of 1945 and spring of 1946 caused salinity depressions from a normal range of 10 to 15 $^0/_{00}$ to zero for protracted periods. Oyster beds located twenty miles south of the entrance of the river into the bay were frequently exposed to fresh water. In the period following these extremes, mortalities up to 70 per cent of the population were recorded on the bars in this area (Engle, 1946). The oysters remaining viable were of unusually poor quality. The body tissues were edematous and nearly transparent. The adductor muscle lacked tonus so that the valves could be separated easily and frequently were gaping.

Samples of ten or more oysters from this low salinity area, designated here as the LS group, were collected weekly in the summer and at longer intervals during the fall and winter of 1946. Transverse sections of the gonad were prepared for histological examination. For comparative purposes, a similar series of oysters, designated as the HS group, was collected in another part of the bay where the salinity was higher and remained relatively unaffected by the flood conditions. These oysters were of good market quality and during the summer produced a set of young oysters of commercial proportions indicating normal gonad development and spawning reactions. Routine hydrographical observations were made at the time of each sampling, as well as plankton tows and notes on the feeding activity and general condition of the oysters.

Of the 185 oysters in the LS group over three years old examined, 40 per cent were females, 33 per cent were undifferentiated, 26 per cent were males and 1 per cent were sex reversals. Of the 221 specimens in the HS group, 70 per cent were females, 29.5 per cent were males and 0.5 per cent were hermaphroditic. The absence of undifferentiated gonads in the HS group was striking in comparison with the LS group.

The orderly sequence of events in the development of functional gametes in the American oyster has been described (Coe, 1932; Loosanoff, 1942), and the résumé of the stages given here for the HS oysters growing in Chesapeake Bay differs in no important respect from conditions found elsewhere except with regard to timing (Loosanoff and Engle, 1940). Spawning is initiated when water temperatures rise to levels approximating 18 to 20° C., and consequently its occurrence varies from year to year at any particular geographical location. Typically, after the final spawning of the population in late summer, there is a short period of rest in which the gonadal tissue is made up of undifferentiated gonial cells. These soon proliferate and early maturation takes place. By this time, usually late December in Chesapeake Bay, water temperatures have decreased to the extent that the oyster becomes inactive and the gonad remains quiescent until the following March. Thus in early spring, gonad sections from the HS oysters are characterized by fairly large numbers of auxocytes. As water temperatures increase, differentiation and growth proceed at a rapid pace, and mature gametes first appear in May when spawning may begin. In June most of the gonads are filled with ripe sexual products, and from that time until early September, successive waves of spawning may continue. By the end of September the majority of gonads are in the resting condition.

In contrast to this typical picture, section of the gonads of the LS group revealed that 5 to 40 per cent of each sample contained gonads which were in the resting

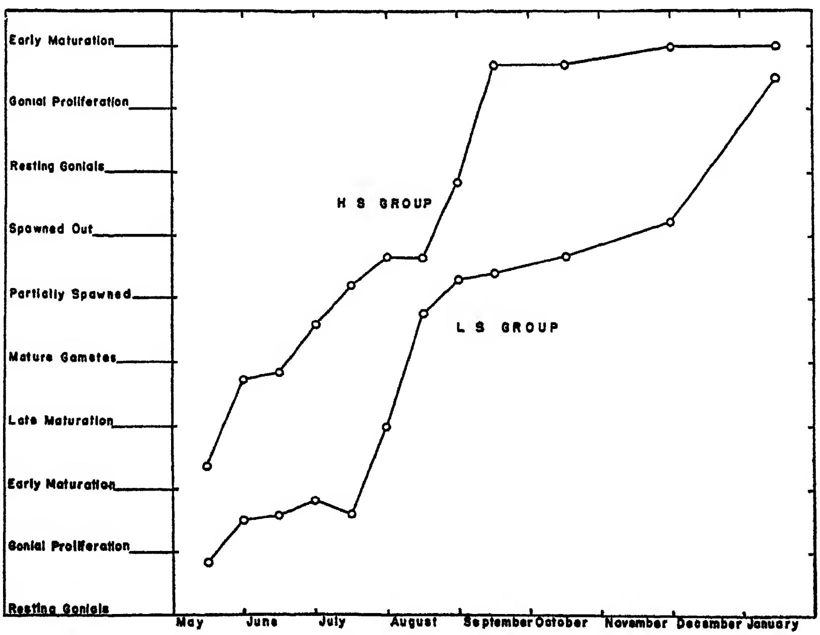

FIGURE 1. Seasonal progression of stages in the development of gametes in oysters from a low salinity area (LS Group) and from a higher salinity area (HS Group). Each point represents the predominant activity in a sample of 10–40 oysters collected during a two-week or longer period. Initial and final phases in the growth of auxocytes, termed Early and Late Maturation, are normally separated by the winter hibernating period in this area.

gonial or undifferentiated stage until the middle of August. This condition must have persisted from the close of the spawning period of the previous year, the time of its normal occurrence. By the end of August there was a marked improvement in the appearance of the oysters, and the gonads reached stages of activity which had characterized the HS oysters examined two months earlier. In early November, a majority of the LS oysters were spawned out, and from this time until January, early maturation continued at a high level. When the oysters finally entered the hibernating stage at temperatures of less than 5° C., the majority of LS oysters were indistinguishable, with respect to the histology of the gonad, from the oysters living in the higher salinity area.

In about 90 per cent of the specimens of LS oysters examined, the gametogenic cycle lagged approximately two months behind that of the high salinity group, but in the remaining 10 per cent of the specimens, the gametogenic cycle showed the same timing pattern as in the HS group. In order to portray graphically the differences between the two populations, the successive stages in normal gonad activity were assigned arithmetic values from one to ten, depending on the preponderant condition or cell type present. The average arithmetic value was then obtained for each sample of gonads collected over a two-week or longer period and has been plotted against time for the two areas studied (Fig. 1).

It was observed that during the summer, developmental stages in different gonads of a sample overlapped or were concurrent because of the relatively long period of four months in which eggs are produced. The average values shown in Figure 1 demonstrate the seasonal trend of the gametogenic cycle, but they do not show the wide variations found within each of the samples of oysters. Earlier investigators (Nelson, 1928; Loosanoff, 1942) have noted the variations in gonad response found in some individuals of a sample where, for unknown reasons, maturation may be delayed or physiologically mature gametes may be retained long after the general population has spawned. This condition is especially prominent in the LS oysters examined. In the first week of August, individuals from one sample demonstrated all stages in gonad development from the undifferentiated gonial cells to the spawned-out stage. The degree of variation among individuals was far less extensive in the HS group, in which for the same period the gonads were fairly equally divided between the partially spawned and the spawned-out stages. The percentage distribution of each stage within the samples collected is tabulated to illustrate this disparity in the two populations (Fig. 2).

During the first two weeks of August there was a significant change in the appearance of the gonad sections from the oysters of the LS group. Wide variations in the stages of activity attained by the individual oysters continued, but all of the gonads suddenly advanced beyond the indifferent and early maturation stages, and 50 per cent of them were partially or almost completely spawned. In this period there were only minor fluctuations in the temperature but the salinity rose abruptly from less than 3 $^{0}/_{00}$ to more than 8 $^{0}/_{00}$. No other environmental changes of importance were noted during this period.

The recovery of oyster larvae from the plankton tows made at the two stations corresponds, in general, with the histological picture.[1] In the high salinity area,

[1] The writer is indebted to Mr. James B. Engle of the U. S. Fish and Wildlife Service, who provided the data on plankton.

larvae were found two weeks after mature gametes were observed in the sections. The last plankton sample taken, October 8, still contained numerous larvae, although it was from two to three weeks after the apparent absence of gametes from the gonad sections. There were two seasonal peaks in larval production: the first week of July and the last week of August. In the low salinity area there was but one seasonal peak toward the end of August. No larvae at all were collected here until seven weeks after their initial appearance in the HS area, although throughout this period 10 per cent of the gonad sections had contained apparently mature gametes. The failure to find larvae in the water at that time may be attributed to inadequate sampling methods or, more probably, to the inhibition of spawning. The observed tissue edema may have interfered with the activity of the adductor muscle in the spawning reaction (Galtsoff, 1938) or have partially closed the gill ostia, thus preventing the passage of ova to the exterior (Hopkins, 1936).

	MAY 16-31		JUNE 1-15		16-30		JULY 1-15		16-31		AUGUST 1-15		16-31		SEPTEMBER 1-15		16-30		OCT		NOV & DEC		JAN	
	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H
EARLY MATURATION																25	9	74	11	75	10	100	55	100
GONIAL PROLIFERATION																5		21	11	20	30		45	
RESTING GONIALS														4	9	10	9	5		5				
SPAWNED OUT								10		20	16	63	30	55	9	50	9		11		20			
PARTIALLY SPAWNED							6	37		80		37	20	41	82	10	55		56		40			
MATURE GAMETE		11	7	75	16	84	12	53	24		47		50				18		11					
LATE MATURATION		39	7	25		16																		
EARLY MATURATION	32	39	36	4	32		35		21		5													
GONIAL PROLIFERATION	21	11	29		26		41		21		5													
RESTING GONIALS	47		21		26		6		34		27													

FIGURE 2. The percentage distribution of different stages of gonad activity in each sample of oysters collected from the low (L) and high (H) salinity areas. See legend under Figure 1 for description of samples.

Water temperatures throughout the period of observations were normal for the region (Fig. 3). In the low salinity area, the bottom temperature was 16.8° C. in the middle of May, approached 20° C. in the first week of June, and reached the summer maximum of 26° C. on the first day of August. It then decreased gradually to 15.8° C. at the end of October, and to less than 5° C. in the period December through January. Bottom water temperatures in the high salinity area regularly followed the same levels within one or two degrees.

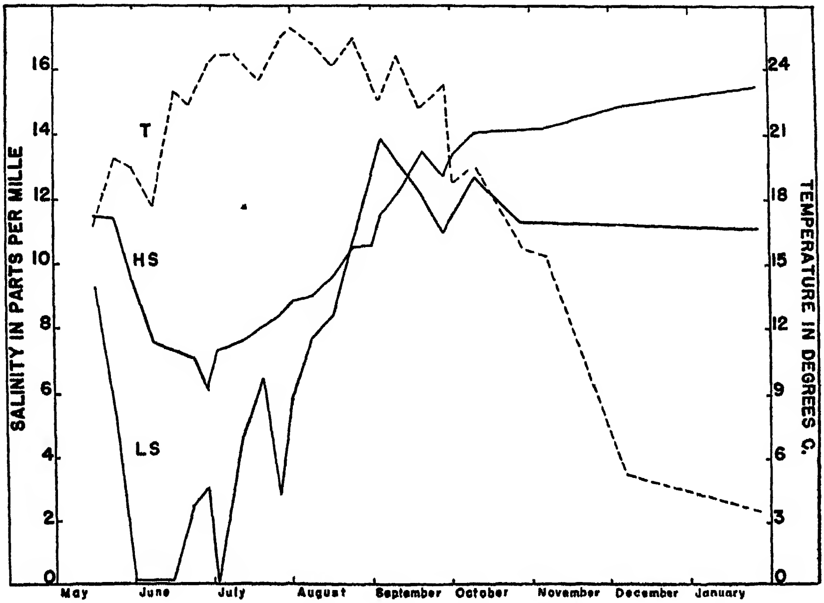

FIGURE 3. Seasonal fluctuations in salt content of bottom waters in the high (HS) and low (LS) salinity areas. The bottom temperature curve (T) is shown for the LS area. Temperatures in the HS area did not vary more than one degree plus or minus from these data.

The salt content of the water was more variable (Fig. 3). In the LS area, bottom salinities fluctuated from zero (fresh) to 6 $^0/_{00}$ in the period from the middle of May until the first of August. One-third of the records for this time showed fresh water. In August the salt level increased steadily to 13 $^0/_{00}$ and then gradually dropped to 11 $^0/_{00}$ by the end of the year. In the HS area, the lowest salinity of 6 $^0/_{00}$ was recorded toward the end of June. Before and after that time, the salinity increased steadily to 15 $^0/_{00}$.

Only four specimens, one per cent of the total examined, gave evidence of the instability of the sex mechanism in this species of oyster. Three of the individuals were clearly defined protandric reversals in which the gonaducts contained residua of spermatozoa, and the walls of the follicles were lined almost exclusively with oocytes in early stages of maturation. These specimens were obtained early in September, which indicates that reversal of sex had taken place when the majority of the population were spawning. Loosanoff (1942) has suggested that sex

reversal takes place when the gonads are made up of undifferentiated gonial cells, usually in late October in Long Island Sound. It would appear most reasonable that sex reversal should take place during this stage, but the specimens found here suggest that if it does, the indifferent stage may occur much earlier in the summer, i.e., July and August, in at least part of the population. In one of the four specimens mentioned above, the gonadal tissue was made up of fairly equal numbers of developing oocytes and spermatocytes within each follicle. The developmental stages attained were the same as in other unisexual specimens collected at the same time, indicating that this oyster would have been a functional hermaphrodite when the general population spawned. The percentage of intersexes found in this rather small sampling agrees with observations by other workers. In the 221 oysters from the HS area there was a ratio of females to males of 2.41. This figure is comparable with observations in Galveston Bay, Texas, but contrasts with the approximately 50–50 sex ratio found along the Atlantic Coast (Hopkins, 1931).

The deleterious effect of the environment on the physiology of the oyster, as evidenced by the delayed production of gametes until such time that the water temperatures made their survival improbable, would appear to be due to the low salinity of the water. That this effect was not a direct inhibition of gametogenesis is indicated by the fact that 10 per cent of the LS group elaborated mature gametes at the usual time in the early summer. The factors directly affected by lowered salinities which may be operating here to prevent gametogenesis include several possibilities. It has been shown (Hopkins, 1936) that during exposure to fresh water the oyster's valves may be closed most of the time and also that even when open, the passage of water through the gills may decrease or stop entirely. Either one or both of these factors would seriously curtail the feeding of the animal. It is also possible that during this time necessary food elements were absent from the plankton, or that tissue edema prevented the normal assimilation of food. In any event the end result appears to have been, fundamentally, a tissue starvation. Hopkins (l.c.) theorized such an end result after studying the feeding mechanism in *O. gigas* in the presence of artificially lowered salinities. It was noted that in the small group of LS oysters which produced gametes at the usual time in late spring, there was a moderate reserve of stored food which gave the tissues a typical opaque appearance. These oysters, as well as the ones having no visible food storage, had empty digestive tracts at the time of examination. This would indicate that the reserve food had been held over from the previous fall rather than that this small group had been able to continue feeding during the period of lowered salinities. The evidence is clear, moveover, that soon after the salinity level rose above 6 $^0/_{00}$ in the first week of August, the animals commenced feeding, there was an obvious improvement in the appearance of the tissues, and gonad activity started to approach the normal picture.

Summary

Histological examination of oyster gonads from an area naturally exposed to prolonged periods of fresh water, when compared to oyster gonads from an adjacent, unexposed area, showed:

1. Gametogenesis was inhibited in 90 per cent of the surviving population until salinity levels rose above 6 parts per thousand.

2. Following the salinity increase, oysters rapidly improved in condition but required from three to four months to attain the same final level of gonad activity as the unaffected group.

3. Marked variation and suppression of gonad activity in the exposed oysters is attributed to variations in food availability, rather than to direct inhibition of sexual activity by less saline water.

4. Sex ratios and extent of intersexuality in the population sampled, as well as details of the gametogenic cycle, agree for the most part with published observations on *Ostrea virginica* in other parts of its geographical range.

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NUCLEAR AND CYTOPLASMIC INTERRELATIONS IN THE FERTILIZATION OF THE ASTERIAS EGG

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The existence of a functional relation between nucleus and cytoplasm is generally accepted, but there are relatively few instances in which the relationship can be demonstrated experimentally. Among ova an extreme case is that exhibited by the maturing ovum. Fol (1877, 1879), in his classic work [1] on the maturation and fertilization of the egg of *Asterias glacialis*, was probably the first to associate the maturation of the ovum with the breakdown of the germinal vesicle. In general, the significance of maturation of the ovum has been too closely limited to the elimination of the polar bodies. More attention should be given to what is probably the basic feature of the phenomenon, namely, changes incurred in the cytoplasm through the admixture of nuclear material from the enlarged germinal vesicle of the ovarian egg (cf. maturation cytoplasmique of Delage, 1901; and R. Chambers, 1921). It has been recently proposed (R. Chambers, 1949) that the cytoplasm of the maturing and mature egg be termed *karyocytoplasm*.

There is also to be considered a relationship between the male and female nuclear elements of the fertilized egg and of both elements with the maturing karyocytoplasm. Many observers have ascribed the movements of the male and female pronuclei to their mutual attraction across the intervening cytoplasm of the egg. An early attempt at testing the existence of such an attraction was made by George Lester Kite, a pioneer in microdissection. In a lecture (unpublished) given during the summer of 1915 at the Marine Biological Laboratory, Woods Hole, Dr. Kite described his efforts at interposing the tip of a microneedle as an obstacle between the male and female pronuclei in the transparent egg of Lytechinus, then known as Toxopneustes. As he dramatically stated: "The pesky nuclei insisted in slipping around the obstacle and no efforts, short of destroying the egg, could prevent the nuclei from approaching one another and uniting."

More recently, E. L. Chambers (1939) was able to offer an interpretation in

[1] Fol's 1879 paper is extraordinary for the abundance and accuracy of his extended observations on the living Echinoderm egg. His assumption of extruded cytoplasmic filaments of the Asterias egg which serve to draw the blunt-nosed spermatozoa through the surrounding jelly to the surface of the egg had been largely discredited until fully substantiated many years later not only for Asterias but also for many of the Asteroidea. Even in regard to the quadrille des centres described by Fol in his paper of 1891 and attacked by Wilson and Mathews (1895), Fol had a case. In his 1879 paper (p. 210) Fol remarked that in heavy polyspermy the sperm asters assume identical distances from one another placed with their centers along a theoretical circle. This fits in with the findings of E. L. Chambers (1939) regarding the sperm aster as a growing spherical gelated body. Several sperm asters simultaneously growing in size would assume the positions ascribed to them by Fol. Such symmetrical positions of four sperm asters would explain Fol's quadrille des centres. It was unfortunate that Fol was not able to correct his one wrong hypothesis because of his untimely death soon after publication of his paper.

terms of physical changes in the cytoplasm. He showed that the movements could be ascribed to the growing sperm aster as a gelated body (R. Chambers, 1917), the sperm pronucleus lying in or close to the center of the aster. The progressive increase in size of the aster transfers the sperm pronucleus passively to a central position in the egg, while the egg pronucleus is carried to the sperm pronucleus by centripetal streaming in radial channels converging at the center of the aster. Fol (1879, pp. 105 and 194), who first described the aster, had already presented the idea that the astral radiations are due to streams of centripetal flow.

The normal dissolution of the germinal vesicle of the fully grown oocyte initiates a gradual and prolonged process (R. Chambers, 1921) which converts the somatic cytoplasm of the ovarian egg into the karyocytoplasm of the maturing egg ready for fertilization. The experiments described in this paper, a brief account of which has been published (R. Chambers and E. L. Chambers, 1940), present the matter in detail with evidence concerning hitherto unsuspected causal interrelations between the egg nucleus, the sperm pronucleus, and the egg cytoplasm during and after alteration of the cytoplasm by the spontaneous dissolution of the germinal vesicle. These interrelations constitute, as it were, the performances of a three ring circus in the maturation of the egg.

The experiments stress features which are concerned with the egg and sperm nuclei during their earlier stages before the sperm aster has attained full expression. They are not to be compared with the egg fragmentation studies of Delage (1899), Tennent, Taylor and Whitaker (1929) and Whitaker (1928), all of which were done on fully mature sea urchin eggs and with reconstituted female pronuclei, both polar bodies already having been eliminated.

Material and Methods

The starfish egg is admirably suited for the present study, since, commencing with the germinal vesicle stage, the eggs develop in sea water and insemination can take place at any time.

Fol (1877) had observed that the eggs of *Asterias glacialis* normally are expelled into the sea water with the germinal vesicle still intact. In our work the fully grown germinal vesicle eggs were uniformly obtained by removing the ripe ovaries into finger bowls of sea water, where the eggs were immediately distributed in a large volume of sea water. Most of the work was done during the months of June and July. Only those batches of eggs were used in which over 90 per cent of samples of the eggs matured. All the bisecting operations on the eggs were done under oil and water immersion objectives.

The fragmented eggs and their controls were maintained at a temperature of 16° C. in syracuse watch glasses. The operations and observations were made in hanging drops suspended from a coverslip in the moist chamber of a micromanipulator at room temperature. The eggs were transferred to the moist chamber, and several eggs immediately bisected. This required about three or four minutes. The eggs were then replaced in the watch glasses at 16° C., kept there until a few minutes before appearance of the sperm aster was expected, and then re-transferred to the moist chamber for observation.

The bisections were performed on the eggs at varying intervals after dissolution of the germinal vesicle, some before and others after insemination. The eggs,

suspended from the roof of the moist chamber, were divided by compressing them with the horizontal shaft of a slender microneedle. The vitelline membrane of the unfertilized egg and the enveloping membrane of the fertilized egg are firm enough to remain more or less intact during the bisection. The two egg fragments, which immediately round up and are completely separated, tend to remain together. The cutting was generally done so as to have both fragments of about the same size, one fragment never being smaller than about one half the volume of the other. Such a difference in size had no appreciable effect on the time of appearance of the polar bodies or of the sperm aster. This is in accord with Tennent, Taylor, and Whitaker (1929) who had shown that the cleavage time of egg fragments is independent of size as long as the fragments, when fertilized, undergo segmentation.

In all the experiments, every individual fragment was kept under observation simultaneously with its companion fragment in the same microscopic field. Hence, when a phenomenon was detected in one fragment it could be immediately compared with what might appear in the companion fragment. The time sequences and the phenomena looked for in each individual case were so clear-cut that intervals as short as two minutes were significant. The phenomena observed were the appearance in the granular cytoplasm of a diminutive radiating star which represented the sperm aster, and the elevation of a hyaline nipple on the surface of the egg, the beginning of one or other of the polar bodies.

Bisecting eggs with intact germinal vesicles confirmed the already recognized finding that fragments lacking the germinal vesicle are not fertilizable (Delage, 1901). After normal dissolution of the germinal vesicle, both fragments are capable of being fertilized, one with a diploid (sperm and egg), and the other with a haploid (sperm) nucleus.

The bisection of eggs already inseminated was done at varying times prior to first polar body formation. As was to be expected, only those fragments were capable of further development which contained the sperm pronucleus. Special attention was given to those eggs in which the sperm and egg nuclei were separated, one in each fragment.

Results

The investigation is classified under two general headings. The first deals with observations on the sequence of events in whole eggs, and the second with bisected eggs. In the latter, attention was directed toward the reactions of the male and female nuclei when together and when isolated in the respective fragments of karyocytoplasm.

I. Observations on the Whole Egg

A. The unfertilized egg

The first intimation of the dissolution of the germinal vesicle is the development of an irregular contour of the membrane and a fading from view of the prominent nucleolus. An irregularity in shape of the membrane is not necessarily related to impending dissolution of the germinal vesicle. A mere collapse of the membrane induced by shaking the eggs does not accelerate maturation. The one

visible change which consistently heralds dissolution is the disappearance of the nucleolus. This is followed by disappearance of the nuclear membrane and a diffusion of the nucleolar contents mixed with the hyaline karyoplasm of the nucleus into the granular cytoplasm. Within five to fifteen minutes, the region formerly occupied by the germinal vesicle is filled with cytoplasmic granules indistinguishable from the rest of the egg. In the granular cytoplasm it is possible to detect the diminutive, hyaline egg nucleus which later gives off the polar bodies.

TABLE I

Sequence of events in the maturing unfertilized eggs of Asterias forbesii at 16–18° C.

	Time
From time of deposition in sea water to:	
Disappearance of nucleolus (50% completion)	8′– 19′
Formation of 1st polar body (50% completion)	76′– 90′
Formation of 2nd polar body (50% completion)	105′–119′

Table I gives the approximate times of the three most obvious events during the maturation of the unfertilized egg. The data were obtained from ten separate batches of eggs of at least 100 eggs in each, kept at a temperature of 16–18° C. The variations in the times recorded are due to the different batches. Within a single batch the variations did not exceed two to three minutes. The figures to the left denote the times, within a two minute range, recorded for seven of the batches. The figures to the right are of one batch. The times for the two remaining batches lie in between.

B. The fertilized egg

Table II, with data averaged from records of five batches of eggs, presents an analysis of the effect on the appearance of the first and second polar bodies and of

TABLE II

Effect of insemination on time of 1st and 2nd polar body formation, and of 1st cleavage in eggs of Asterias forbesii, at 16° C.

(1) Eggs inseminated at following intervals of time after deposition in sea water:	(2) Time 50% 1st P.B. formation after deposition in sea water:	(3) Time 50% 2nd P.B. formation after deposition in sea water:	(4) Time 50% 1st cleavage after deposition in sea water:	(5) Time 50% 1st cleavage after 2nd P.B. formation:	(6) Time 50% 1st cleavage after insemination:
—	78.2 (unfertilized)	107.3 (unfertilized)	—	—	—
25′	70.0	98.0	169.0	70.5	144.5
40′	71.5	101.0	172.0	71.0	132.0
50′	74.0	102.5	173.0	70.5	123.0
60′	75.8	104.0	175.0	71.0	115.0
70′	77.5	105.5	178.0	72.0	108.0
80′	—	106.5	184.5	78.0	104.5
90′	—	107.0	193.5	86.5	103.5
100′	—	107.5	203.5	96.5	103.5
130′	—	—	234.0	127.0	104.0

the first cleavage by inseminating the eggs at successive intervals following breakdown of the germinal vesicle. The first column gives the times of insemination. The figures in the second and third columns show that, up to a certain time, the earlier the insemination the more accelerated is the formation of the polar bodies. When the insemination is delayed to and beyond the time of first polar body formation, there is no evidence of acceleration, whereupon, the time of appearance of the second polar body tends to coincide with that of its appearance in the unfertilized egg.

Evidently it is only when the fertilization process is started early that the conversion of the egg nucleus into its pronucleus is accelerated. Later, when fertilization occurs at the time that the polar body formation has been initiated, there is no longer any appreciable accelerating action.

A consideration of the cleavage times, presented in the fourth, fifth and sixth columns, brings out several significant features. From the fourth column it can be seen, as is to be expected, that cleavage time corresponds with the time the eggs are deposited in sea water. However, during the earlier stages, up to some time before first polar body formation (after 60 minutes), the lapse is not as great as during the later stages (cf. Fig. 1). This is brought out more clearly from the figures in the fifth column which give the times between those of first cleavage and of second polar body formation. They indicate that the time interval, irrespective of insemination time, is constant until about the time when the first polar body is being initiated. After this the cleavage time becomes directly proportional to the insemination time.

The figures in the sixth column give the times between insemination and first cleavage. They show that the earlier the insemination up to the time when the first polar body is initiated (about 70 minutes), the longer is the time which elapses before cleavage occurs. After 70 minutes the time between insemination and cleavage becomes constant.

These analyses indicate that the rate at which the fertilization events proceed depends upon the cytoplasmic maturation which is completed at about the time of first polar body formation. Prior to this, it would seem that the immature state of the karyocytoplasm has a delaying effect on the development of the sperm and its accompanying events. Upon initiation of first polar body formation, the maturation of the karyocytoplasm is complete, whereupon the development of the sperm from the time of its entry proceeds without delay and cleavage occurs within a constant period of time.

A graphic presentation of Table II is given in Figure 1. The abscissae represent the times of insemination; the ordinates, the times when the various events occur. Concerning the unfertilized egg, the two vertical dotted lines and the two horizontal dotted lines intercept the X and Y axes respectively at the times when the first polar body forms (average of 78.2 minutes) and when the second polar body forms (average of 108.2 minutes).

Concerning the fertilized egg, the three solid curves represent the times for the formation, respectively, of the first and of the second polar bodies, and of the first cleavage in eggs inseminated at different intervals after germinal vesicle breakdown. The curves for the first and second polar body formation are parallel throughout and their upward slopes represent the acceleration due to insemination.

It is to be noted that when the insemination occurs at 78 minutes (time of first polar body formation) or later, the time of second polar body formation remains the same as that of the unfertilized egg.

Let us now consider the dotted dash curve which represents the time of first appearance of the sperm aster and which was calculated from data obtained on about 100 eggs observed with an oil immersion objective. The sperm aster never appears until after the second polar body, no matter how early the eggs have been inseminated (the earliest recorded being at 25 minutes). During these earlier

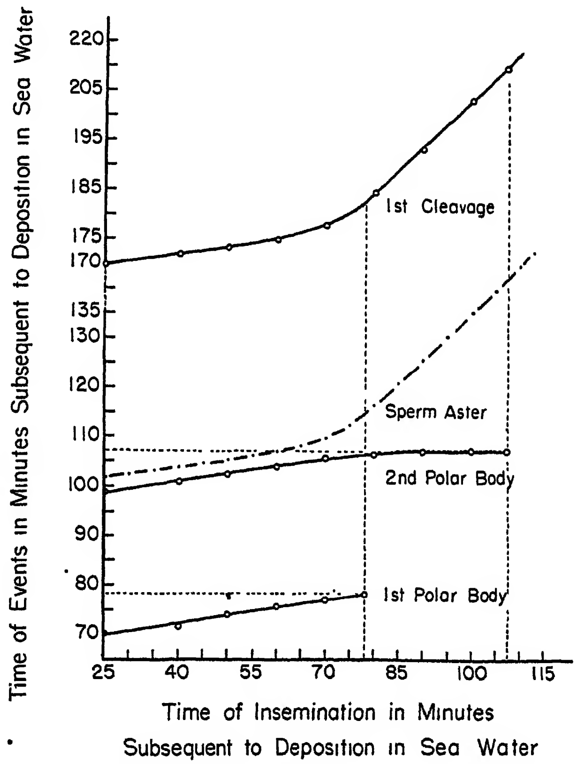

FIGURE 1. Relation of time of insemination to time of appearance of first P.B., second P.B., and first cleavage (solid lines), and of sperm aster (dotted dash line). Faint dotted horizontal lines represent times of appearance in unfertilized eggs of first and second P.B. The observations were made on eggs of *Asterias forbesii* at 16° C.

stages of the developing egg, it appears at two to three minutes after the second polar body has been given off. In later stages, viz., after 70 minutes, this interval becomes progressively greater. It is to be noted that the interval between the time of appearance of the sperm aster and the time of first cleavage is always just about 68 minutes, whether the time of insemination is early or late. This is the normal time for the events following aster appearance. The length of the interval between the time of insemination and the time of sperm aster appearance varies, depending upon the state of maturation of the karyocytoplasm. It becomes con-

stant only when the egg is fully mature, which also coincides with the time of first polar body formation. Thus, when the eggs are inseminated as early as 25 minutes after being placed in sea water, a period of about 78 minutes must elapse before the sperm aster appears. When the egg is inseminated during or after the first polar body formation, the interval between insemination and appearance of the sperm aster is found to be constantly about 35 minutes.

Insemination after formation of the first polar body results, as Lillie (1915) has shown, in a tendency toward a decline of fertilizability and of subsequent development. Abnormalities become pronounced when eggs are inseminated 30–60 minutes after the formation of the second polar body.

To summarize: there is a period of progressive ripening of the karyocytoplasm. Optimum ripening is heralded by the development of the polar asters and the initiation of the sperm pronucleus to form its aster. After this period there is a decline in the proper functional interrelations between the sperm and karyocytoplasm. The decline is made evident by the fact that sperm entry, subsequent to second polar body formation, results in an increasing abnormality of cleavage.

II. Observations on Bisected Eggs

A. Unfertilized eggs bisected and the fragments immediately inseminated

1. **Early bisections up to about ten minutes before first polar body formation.** Thirty pairs of fragments were studied. The cutting was done at 25, 40 and 60 minutes after deposition of the eggs in sea water (ca. 10, 25 and 45 minutes respectively after the germinal vesicle had disappeared). Each pair of fragments was then inseminated immediately. Figure 2 is representative of all the cases. The sperm aster in the non-egg-nucleated fragment appeared earlier than in the egg-nucleated fragment. Its time of appearance was always *after* the companion fragment had formed its first polar body and two to three minutes *before* the formation of the second polar body. On the other hand, in the egg-nucleated fragment, the sperm aster never appeared until two to three minutes after the second polar body had been formed. This difference between the two fragments was reflected in the earlier cleavage of the haploid fragment.

2. **Late bisections immediately before and during first polar body formation.** Twenty pairs of fragments were studied. In all of them the sperm aster appeared simultaneously at about two to three minutes after formation of the second polar body in both non-egg-nucleated and egg-nucleated fragments of each pair. The cleavage time of both fragments was simultaneous.

B. Eggs fertilized early and fragmented at varying times until shortly after first polar body formation

The eggs were inseminated 25 minutes after deposition in sea water, that is, shortly after dissolution of the germinal vesicle. In many of the bisected eggs both male and female nuclei lay in the same fragment. These double nucleated fragments, regardless of the time of cutting, behaved exactly like the whole eggs in regard to the time of polar body formation, appearance of the sperm aster and subsequent cleavage. Attention was devoted to the few fragments in which the

sectioning had separated the sperm from the egg nucleus. Four fragments were of eggs cut at 35 minutes; three at 50 minutes, and six at 74 minutes after deposition in sea water.

The results are shown in Figure 3. In the eggs (A_1) cut 35 minutes after deposition in sea water, the sperm aster (A_2) appeared after the first polar body of the companion fragment and two to three minutes before the second polar body. In an egg (B_1) cut at 50 minutes, the sperm aster (B_2) appeared simultaneously with the second polar body of the companion fragment. In an egg (C_1) cut at 74 minutes, the sperm aster (C_2) appeared after the second polar body in the companion fragment. D represents the first cleavage stage, at 170 minutes, of the eggs, A, B, and C. Cleavage occurred, as is to be expected, only in the fragment containing the sperm pronucleus. The egg nucleus in the other fragment produced the first and second polar bodies at the same rate as that of fertilized control whole eggs and, finally, moved to a central position in the fragment, where, as the female pronucleus, it enlarged somewhat but otherwise remained quiescent. The time of appearance of the polar bodies was thus seen to be the same, irrespective of when the sperm pronucleus had been separated from the egg nucleus by the cutting process. Evidently neither a brief nor a long sojourn of the sperm pronucleus in cytoplasmic continuity with the egg nucleus affects the hastening which the fertilization process induces in the formation of the polar bodies.

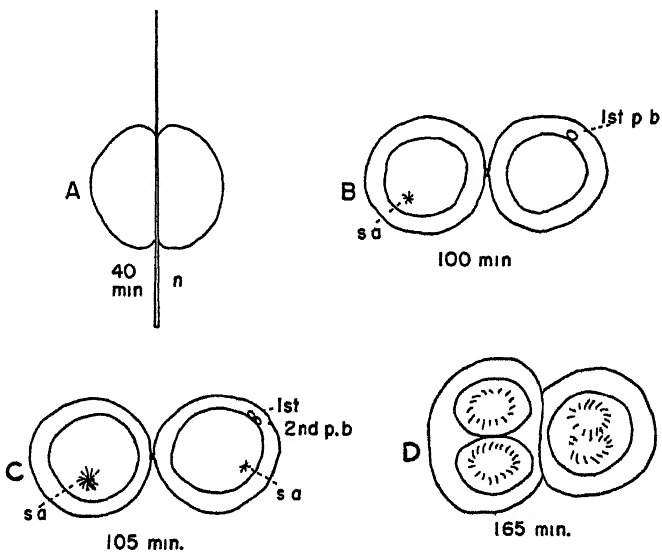

FIGURE 2. Asterias egg bisected 40 minutes after deposition in sea water, both fragments inseminated simultaneously.

A. At 40 minutes. Cutting of unfertilized egg with microneedle, *n*.

B. At 100 minutes. Both fragments with fertilization membranes. Haploid fragment with sperm aster, *s.a.* Diploid fragment with first P.B. which had formed 25 minutes earlier.

C. At 105 minutes. Haploid fragment with considerably enlarged sperm aster. Diploid fragment with beginning sperm aster and second P.B. which had formed two minutes earlier.

D. At 165 minutes. Haploid fragment just after completion of first cleavage. Diploid fragment still in amphiaster stage.

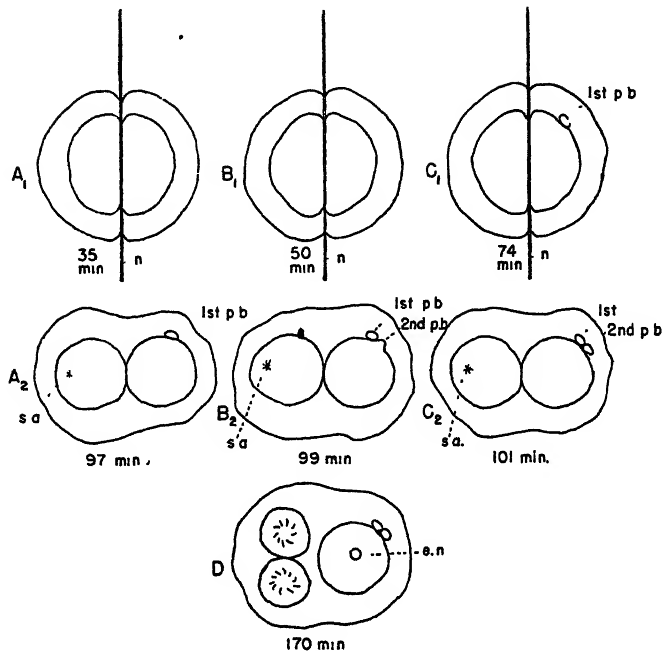

FIGURE 3. Three eggs inseminated 25 minutes after deposition in sea water and then bisected at different times so as to have sperm pronucleus in one fragment and egg nucleus in the other.

A. Cut at 35 minutes (10 minutes after insemination), A_1. At 97 minutes, A_2. Beginning sperm aster, *s.a.*, appears in one fragment and first P.B. in companion fragment.

B. Cut at 50 minutes (25 minutes after insemination), B_1. At 99 minutes, B_2. Beginning sperm aster appears in one fragment and beginning second P.B. in companion fragment.

C. Cut at 74 minutes, just after the first P.B. has formed, C_1. At 101 minutes, C_2. Beginning sperm aster appears in one fragment and completed P.B. in companion fragment.

D. Condition of all three bisected eggs at 170 minutes. Sperm-haploid fragment has cleaved, while egg nucleus, *e.n.*, of companion fragment has taken a central position and remained inactive.

DISCUSSION

The results presented in this paper stress two major features concerning the events after the material of the germinal vesicle has mixed with the cytoplasm of the egg. One deals with the maturation of the karyocytoplasm; the other, with the fertilization process of the male and female nuclear elements in their relations to the maturing karyocytoplasm.

Delage (1899) had already surmised that the dissolution of the germinal vesicle is essential to maturation and fertilizability of the sea urchin egg, and confirmed it from his merogonic experiments (1901) on the immature eggs of *Asterias glacialis*.

In our experiments with *Asterias forbesii* the disappearance of the germinal vesicle and the mixing of its hyaline fluid with the granular cytoplasm lasts about 10–15 minutes. The resulting karyocytoplasm contains the definitive egg nucleus.

Any viable fragment of this karyocytoplasm is fertilizable. The significant feature is that the karyocytoplasm must undergo a protracted maturing process. The prime evidence for completed karyocytoplasmic maturation is the appearance of asters initiated in the egg either by the egg nucleus in forming the polar bodies, or by the spermatozoon in forming its sperm aster.

It is of interest to note that when the fertilization process is initiated in eggs with still maturing karyocytoplasm, the activity of the egg nucleus starts *earlier* than it would if the egg were unfertilized.[2] On the other hand, the activity of the sperm pronucleus in forming its aster starts *later* when it is associated with the egg nucleus than it does when it is isolated in a separate body of karyocytoplasm. This indicates that the fertilization process accelerates the egg nucleus to polar body formation, while the presence of the egg nucleus delays the formation of the sperm aster. As an example of this, let us consider the situation in which an egg nucleus and a sperm pronucleus are lying together in karyocytoplasm which is still maturing. Upon completed maturation of the karyocytoplasm, there is initiated around either the male or the female nucleus a localized, centripetal cytoplasmic streaming which becomes evident to the eye as asters. The first asters to appear are those of the polar spindles of the egg nucleus. Not until the second polar body has been eliminated is there any sign of cytoplasmic streaming centered about the sperm pronucleus for the formation of the sperm aster. The course of these phenomena is of phylogenetic interest, viz., the fact that it is the egg nucleus rather than the sperm around which the radial streaming first occurs.

In the development of the sex elements, the last step taken by the fully grown primary oocyte is to undergo two successive cleavages (equational and reductional). In the early history of sex the resulting four egg cells may be equal in size or, in accordance with later evolutionary changes, they may be unequal, viz., the typical egg and its three polar bodies. In either event, growth of the mother cell, followed by two successive nuclear mitoses, has been repeated presumably over countless periods of time before the male sex cell came into being. This would establish a condition such that the maturation of the karyocytoplasm tends to lead directly to the formation of the two polar bodies. The sperm in the egg is a relatively late comer in evolution so that reactions concerned with it should come after, with the development of the sperm aster and eventually the amphiaster of the first cleavage spindle of the fertilized egg. This might be regarded as a case of evolutionary memory, colloidal or otherwise.

When the sperm pronucleus is isolated in a non-egg-nucleated fragment of an egg with karyocytoplasm which has not yet become mature, maturation leads to cytoplasmic streaming and aster formation about the sperm pronucleus. There is no egg nucleus to assert priority, and the result is that the sperm aster appears before it otherwise would.

When insemination occurs after the karyocytoplasm has completed its maturation, the conditions which now exist do not call for an interplay of the reactions described above. In a completely mature egg, the lapse of 30 to 35 minutes be-

[2] Recently, Lovelace (1947) was able by artificial means to accelerate the penetration of the spermatozoan in the Nereis egg. She found that this induced earlier formation of the polar bodies than would have been the case if the sperm had penetrated later. Fol (1879, pp. 117 and 335) had already noted that for the Asterias egg, polar body formation is accelerated by early insemination of the egg.

tween sperm entry and appearance of the sperm aster is just about the time between the initiation of the first and completion of the second polar body. Therefore, if sperm entry occurs at the earliest moment of completed karyocytoplasmic maturation, i.e., just prior to the formation of the first polar body, the astral streaming of the polar body spindles will have been completed before the sperm aster begins to be appreciable.

An indication of the necessity for proper time relations between the formation of the sperm aster and that of the polar body asters is given in extremely interesting experiments performed years ago by A. Brachet (1922). Brachet discovered a means of disturbing these time relations, and by doing so secured abnormal astral configurations. Brachet found that the immature eggs of Paracentrotus, on being removed from the ovary, could be stopped at various stages of their maturation by plunging the eggs into sea water. In the sea water these eggs readily became polyspermic, and the sperm which had entered continued to develop and formed sperm asters. These asters either remained small or grew to larger dimensions according to the stage of the eggs they were in. The stages of special interest in this discussion were those of the eggs possessing egg nuclear polar spindles and their asters. Sperm asters present at the same time became intermingled with them and formed abnormalities such as tripolar mitoses, etc. Fol was also able to observe similar discrepancies in polyspermic Asterias eggs. In the event that several sperm asters appeared while the chromosomal vesicles of the egg nucleus in mitosis were still infused, Fol noted that one or more of the vesicles became incorporated in the sperm asters, thus upsetting the normal course of events.

The avoidance of such a phenomenon is ensured in the Asterias egg, which normally matures in sea water and which is fertilizable at any stage during its maturation. In monospermic eggs an appropriate time-spacing between the male and female nuclear events is occasioned by the following: On the one hand, the formation of the egg-nuclear polar bodies is accelerated by the fertilization process, while on the other, the appearance of the sperm aster is delayed by the presence of the egg nucleus. The two features combine to separate in time the formation of the polar bodies from the formation of the sperm aster. The result is that in the normal course of development, the cytoplasmic streaming, involved in the formation of the polar body asters, reaches completion before the initiation of the streaming associated with the growing sperm aster. It appears, therefore, that the peculiar interrelations between karyocytoplasm, egg, and sperm nuclei are of service in preventing a possible interference between the reactions concerned in polar body formation and those concerned with preparation of the fertilized egg for its first cleavage.

Summary

Full-sized germinal vesicle oocytes of *Asterias forbesii* undergo normal maturation in sea water. At 16° C. the first polar bodies are formed in about 80 minutes, and the second, in 108 minutes. The eggs are sperm-fertilizable from the time of germinal vesicle breakdown until some time after elimination of the second polar bodies. Fol (1879, p. 204) indicated that the optimum time for insemination is after germinal vesicle breakdown up to the first polar body formation. In accordance with Fol, the earliest period for the sperm aster to appear was found to be

always a few minutes after the formation of the second polar body. A feature to be stressed is the progressive change of the karyocytoplasm induced by the mixing of the contents of the germinal vesicle with the cytoplasm during maturation.

Maturation of the karyocytoplasm

1. When eggs are inseminated two to three minutes before first polar body formation or later, the time for the sperm aster to appear in the living egg is about 35 minutes at 16° C.

When the eggs are inseminated at any time prior to the above, the time taken for the sperm aster to appear is equal to 35 minutes plus the interval between the time of insemination and the time of initiation of the first polar body.

Evidently, therefore, the time of appearance of the sperm aster is a function of the maturation of the karyocytoplasm. The maturation begins at the time of germinal vesicle breakdown and reaches completion two to three minutes prior to formation of the first polar body. In a fully mature karyocytoplasm the interval between sperm entry and the appearance of the sperm aster is constant.

The egg nucleus

2. Sperm-fertilization of whole eggs or of egg-nucleated fragments accelerates the egg nucleus in the formation of its polar bodies. The earlier the insemination the greater is the acceleration.

3. The effect of the fertilization process in accelerating polar body formation persists after removal of the sperm pronucleus. This was ascertained by removing the sperm pronucleus, through bisection, at several intervals of time, the earliest being ten minutes after insemination.

In other words, once given the impetus the egg nucleus maintains its hastened progress independently of the presence of the sperm pronucleus.

The sperm aster

4. In eggs bisected while undergoing maturation and then inseminated, the sperm aster appears earlier in the fragment lacking the egg nucleus than in the egg-nucleated fragment.

In eggs fertilized while undergoing maturation and then bisected at different times, the sooner the sperm pronucleus has been isolated from the egg nucleus, the earlier the sperm aster appears.

In other words, the presence of the egg nucleus has a delaying action on the development of the sperm aster. However, the earlier the egg nucleus has been removed through bisection of the egg, the less is the delaying action.

General Conclusion

There is a close interrelation between (a) the fertilization process, (b) the ripening of the karyocytoplasm, (c) the development of the sperm pronucleus, and (d) the activity of the egg nucleus in forming its polar bodies. The fertilization process, by hastening the maturation of the karyocytoplasm, accelerates the activity of the egg nucleus in forming its polar bodies. On the other hand, the egg nucleus

exerts a lag effect on that feature of the maturation of the karyocytoplasm which is concerned with the development of the sperm aster. The net result is the attainment of an adequate spacing between the times of the cytoplasmic streaming activities concerned with polar body formation and those concerned with the development of the sperm aster. This permits normal development of Asterias eggs fertilized at any time during their maturation.

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FORM AND GROWTH IN THE DEVELOPMENT OF A SCYPHOMEDUSA

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The nature and development of the scyphistoma and strobila of certain Scyphomedusae have been described a number of times, from various points of view.

Among the Semaeostomae, our knowledge of Aurelia and Chrysaora (including Dactylometra) is fairly complete, although correlations of form and size have not been emphasized. The other two forms that have been studied to some extent are Pelagia, the egg of which transforms directly into a medusa, and Cyanea. In the case of the Rhizostomae the developmental cycle is known for Cassiopea, Cotylorhiza and Nausithoë.

The present account is based upon a collection of scyphistomae and strobilae tentatively identified as those of *Cyanca capillata* Eschscholtz.

Source of Material

The material was part of an unlabelled collection in the Zoology museum at McGill University, a circumstance that adds an uncertainty of original site to the usual uncertainty of parentage of scyphistomae found in their natural habitat.

Fortunately, the internal evidence is decisive. The scyphistomae were attached to ascidians or to eel grass (*Zostera marina*) to which the ascidians in turn were attached. Fastened between some of the ascidians were several very young specimens of *Cucumaria frondosa.* The presence of the holothurian places the locale on the Atlantic coast north of Cape Cod. The ascidian is definitely identified as *Molgula provisionalis* Van Name, a species closely related to *M. manhattensis* and previously confused with it (cp. Van Name, 1945, p. 389). *Molgula provisionalis,* however, is recorded only from waters in the general region of Eastport, Maine, from Passamaquoddy Bay to Mount Desert. Since it is known that collections of this species of Molgula, attached to eel grass, have been made at St. Andrews Point in Passamaquoddy Bay, there is little doubt that the material is part of such a collection, and in any case there appears to be no doubt that these scyphistomae came from shallow water near the mouth of the Bay of Fundy.

Identification of Material

Identification of the genus and species is rather more difficult. The obvious suspects are *Aurelia aurita* and *Cyanea capillata,* since both of these are abundant in the region. *Dactylometra quinquecirrha* (a "Chrysaora") reaches the shoreline at Cape Cod, but is not reported from inshore waters of northern New England. The most northerly occurring rhizostomid of the Atlantic coast is *Rhopilema verrillii,* a southern form that occasionally strays into Long Island sound. The only

remaining form is *Phacellophora ornata,* another semaeostomid, which is known only from Eastport and the Bay of Fundy as two isolated records, by Verrill in 1869 and Fewkes in 1888.

The strobilae do not resemble those of Aurelia (cp. Percival, 1923) or Chrysaora (cp. Chuin, 1930), and while they are remarkably like those of the rhizostomids Cassiopea (cp. Bigelow, 1900) and Cotylorhiza (cp. Claus, 1892), it is not reasonable to assume the occurrence of an unknown rhizostomid in the region in question, nor to extend the range of Rhopilema from Long Island Sound through the five hundred miles of cold water north of Cape Cod. The alternatives remain Cyanea or Phacellophora, and the absence of any record of Phacellophora during the last sixty years makes it a most unlikely candidate. It is provisionally assumed, therefore, that our scyphistomae and strobilae belong to Cyanea, even though the somewhat brief earlier descriptions of the life cycle of Cyanea are significantly different from the account given here.

The Cyanea of the western Atlantic is *C. capillata* Eschscholtz. According to Mayer (1910), *C. arctica* Perón and Lesueur and *C. lamarckii* Perón and Lesueur are synonymous, or at the most are varieties of doubtful stability. The embryonic and early larval stages have been intensively studied by Hyde (1894) as *C. arctica.* Young scyphistomae were reared by L. Agassiz (1862) as *C. arctica* and by Perez (1920) as *C. capillata.* Planulae were reared in aquaria through the scyphistoma to the strobila and ephyra stages by Hargitt (1902 and 1910) as *C. arctica* and by Delap (1905) as *C. lamarckii.* The scyphistomae described by the above investigators might well be of one and the same species, but the strobilae are very differently described and in neither case do they conform at all closely with the one given here. Both Hargitt and Delap obtained planulae directly from known medusae, and the difference expressed in their descriptions must be due either to differences in culture conditions or to a genetic difference in the parent organisms. These differences will be discussed following the description of the present material, which in spite of the element of doubt will be assumed to be that of *Cyanea capillata.*

Growth of the Scyphistomae

Since there is no indication that long lateral stolons are formed, as in Aurelia, that could produce buds at a considerable distance from a parent scyphistoma, the minute individuals found in scattered and very isolated positions are assumed to be newly attached planulae. The possibility of migratory buds, however, is not excluded. Typical examples are shown in Figure 1, A–D.

The planula apparently attaches by its narrow end, and in some cases at least sends out two or three root-like processes of attachment (Fig. 1, A, B). Four tentacles appear around the developing manubrium, while four more are added, bringing the number to eight without significant change in size from the original state (Fig. 1, C, D). Eight new tentacles appear, raising the total to sixteen, again with little increase in the size of the whole.

At the same time a small bud protrudes from the wall of the hydroid at or near the junction of the body and stalk (Fig. 1E). Similar buds, appearing at the same site, occur in scyphistomae of all sizes (Fig. 1, G–J), although many scyphis-

tomae equally representative of all sizes were found without buds (Fig. 1F). The conclusion is that a series of such buds may be produced by an individual scyphistoma. The first appearance of a bud in a minute scyphistoma is in itself an expression of a local acceleration of growth, and it would be gratuitous to assume that this growth would become abruptly arrested and that the same bud would remain

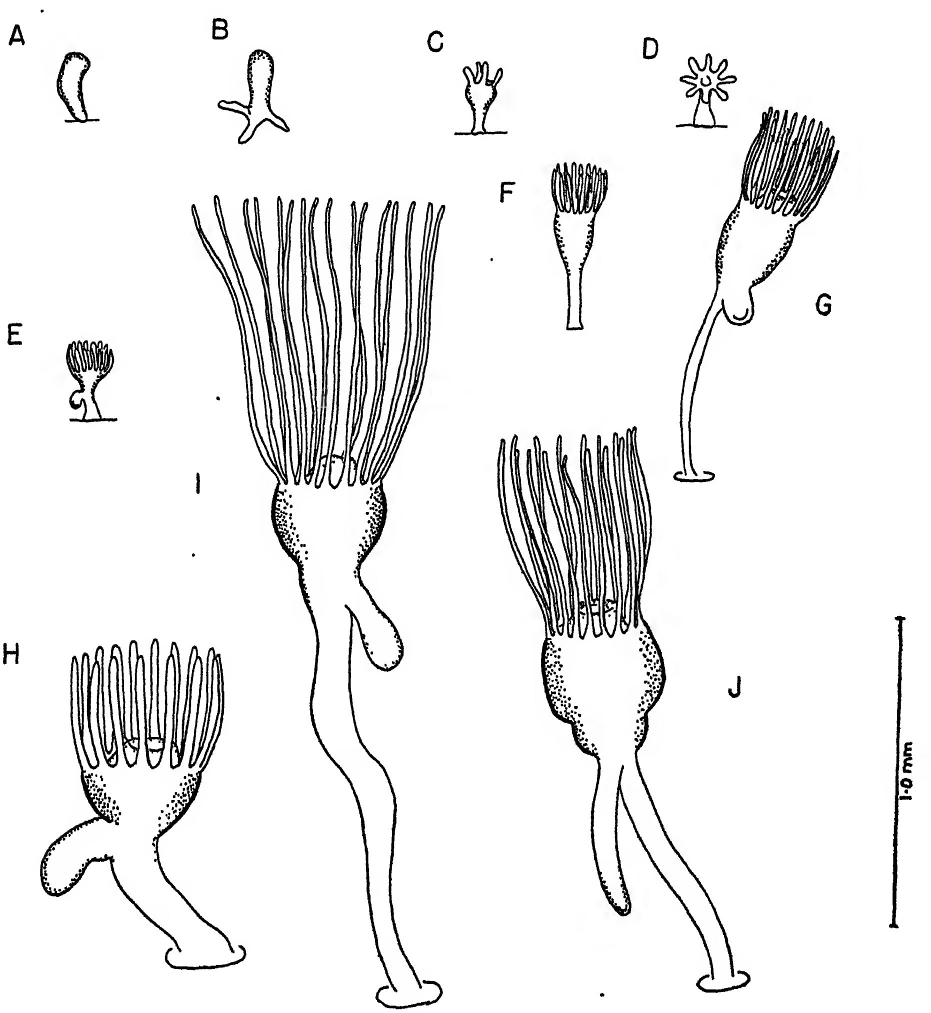

FIGURE 1. Growth and budding of scyphistomae of *Cyanea capillata*. A, B, attached planulae. C, 4-tentacle scyphistoma. D, 8-tentacle form. E, 16-tentacle scyphistoma with lateral bud. F, larger form without bud. G, H, I, J, older scyphistomae with buds.

but little changed in relative proportions in the large scyphistomae. It is more reasonable to interpret the conditions illustrated as being either the production of several buds successively from one site, or the production of but one bud, though at different stages of growth among different individuals.

In the great majority, the direction of growth of the bud is from the top of the stalk downwards towards the substratum. Growth of the bud is primarily stolonic, and is mainly by terminal proliferation of cells (cp. Fig. 1J). The largest scyphistoma of this type is shown in Figure 2B. No indication that such outgrowths extend to any distance has been found, and the occurrence of associations such as that shown in Figure 1, A and E, suggests that the buds grow down to become attached to the substratum close to the base of the parent, and constrict off from the parent at the point of origin.

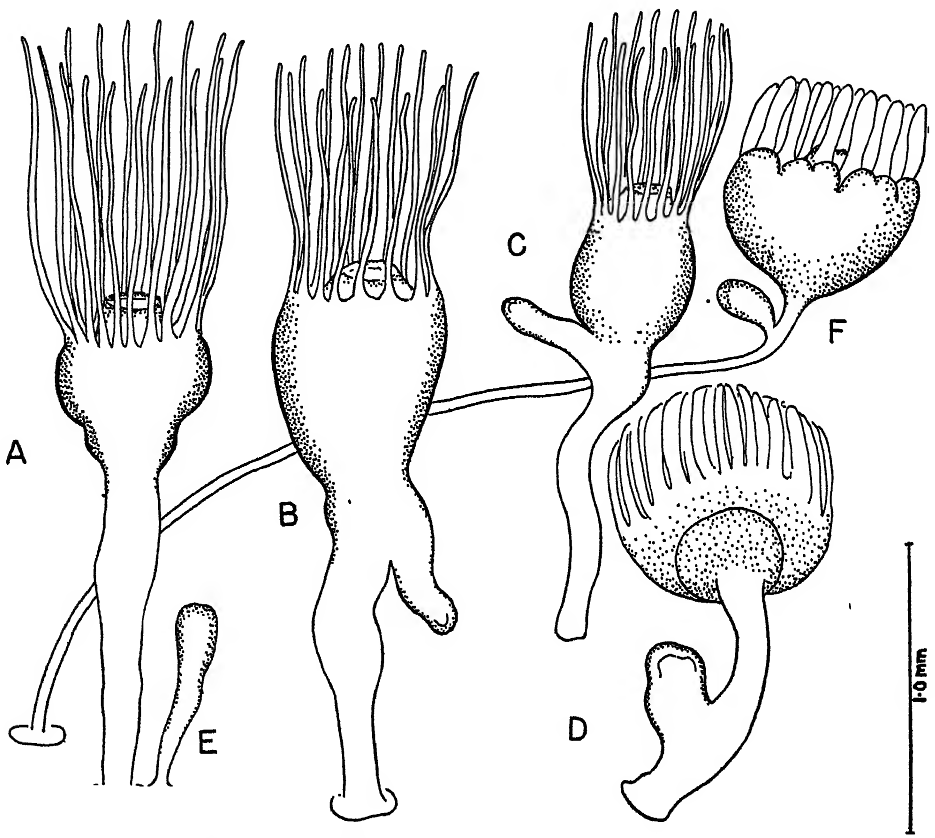

FIGURE 2. Fully grown scyphistomae of *C. capillata*. A, commencement of strobilation B, with bud directed downwards. C, D, with buds directed anteriorly. E, detached and attached bud at base of parent. F, metamorphosing scyphistoma with late bud.

In a minority of cases the bud grew upwards instead of downwards (Fig. 2, C, D) and in one case grew from the top of a long tenuous stalk that was bearing a metamorphosing scyphistoma at its end. Conditions such as these probably lead to those shown in Figure 3, A and B. In fact, Figures 2C and 3A might well be placed in sequence, the scyphistoma of Figure 2C having partially metamorphosed to become an ephyra in Figure 3A, the bud of Figure 2C having become a scyphistoma in Figure 3A, while the mutual relationship of the stalks remains unchanged.

On the other hand, the comparable stages of metamorphosis exhibited by the two heads of the individual shown in Figure 3B suggest the possibility that the division of the distal end preceded differentiation into scyphistomae, especially since the head that is somewhat the smaller is actually the more advanced, for only the eight interlobular tentacles remain. Such a condition seems more likely to arise

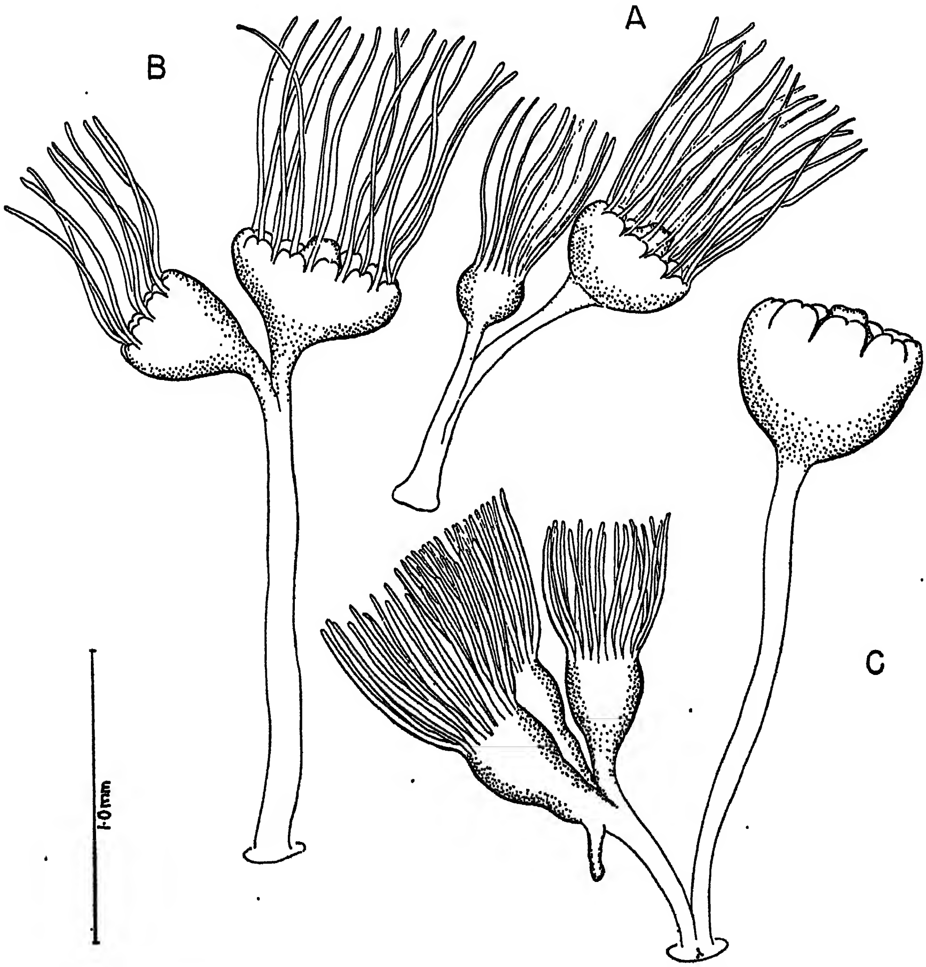

FIGURE 3. Retention and division of buds of *C. capillata*. A, bud forming scyphistoma attached to stalk of parent. B, double-headed strobila. C, strobila with three-headed scyphistoma attached to stalk base.

at the point of detachment of a bud from its parent than at the distal end of a newly attached planula. This is somewhat forcibly indicated by the example shown in Figure 3C. The parent scyphistoma is well advanced in its metamorphosis into an ephyra. The associated stalk may possibly have arisen from a bud similar to that seen in Figure 2D, but one arising even more proximally, or equally, if not more

likely, from a bud that grew downward from the usual site to become attached at the base of the parental stalk. In any case its distal end has given rise to three scyphistomae of approximately equal size. It does not seem possible that any one of the three could have given rise to the other two by budding, for there is too close an identity of size and form. In one of the three individuals a bud is growing downward, almost like a regeneration of an additional stalk to compensate for the multiplicity of heads.

Formation and Development of Strobilae

During the process of growth, the scyphistoma becomes progressively differentiated into stalk and head as in Figure 1I. In many cases metamorphosis into an ephyra occurs in a typical manner and purely as a monodisk. The head shortens and widens, eight of the sixteen tentacles resorb during the formation of the eight rhopalia, while somewhat later the eight interlobular tentacles are also resorbed. At the same time, the outer margin of the scyphistoma divides into eight lobes corresponding to the lappets of the future ephyra.

While in many cases a single ephyra may form from the head of a scyphistoma, in as many others, if not more, two or three ephyrae are produced in series. Whether one or more are to be formed is discernible from the contour of the scyphistoma before there is any other metamorphic indication, as in Figure 1J and 2A. In most cases, if not all, the interlobular tentacles are retained until shortly before the ephyra is set free (Fig. 3A). In no case have tentacles been seen in a developing ephyra that is second in line.

Three stages in the later development are illustrated by Figure 3, A, C, and E, representing the eight-tentacle stage (3A), all tentacles resorbed (3C), and the fully developed ephyra on the point of liberation (3C).

Cases such as the one shown in Figure 3B, in which two ephyra are almost at the same advanced stage of development, suggest that the ephyra probably grows to a certain critical size, when its development is functionally complete and it is ready to be set free, even though greater differences in size may be more evident at an earlier stage (cp. Fig. 3A). The individual shown in Figure 3D probably represents a second ephyra, the first having been liberated, and the same may be true for the primary individual in Figure 2C. Otherwise there is considerable variation in the time or size at which all tentacles become resorbed.

In all of the individuals with ephyrae, shown in Figure 3, there is present a relatively small basal swelling at the junction with the stalk, suggestive of a third ephyra. Marginal lobes tend to develop, though not in relation to any particular size (cp. Fig. 3A, 7D), and it is possible that an ephyra would have developed. The fact, however, that no individual has been found with three unmistakable ephyrae in process of formation may mean one of two things; either the third effort remains abortive, or else the first ephyra is always liberated before the third is definitely established.

It is notable that these third attempts at annular growth usually bear short tentacles in the lobular position (e.g. Fig. 3, C and E), possibly indicative of the re-establishment of the scyphistoma state.

A number of isolated stalks were found, of the same size as the largest bearing ephyrae, which possessed four distal tentacles as in Figure 3F. These may rep-

resent a return to the scyphistoma condition as is generally the case in Aurelia and Chrysaora, giving rise to another crop of ephyrae at some later time. On the other hand no scyphistoma was found that had a fully grown stalk and a head with either eight or sixteen tentacles. In our opinion such stalks as that illustrated are merely the final differentiation of the residual stumps after the ephyrae have been liberated, and in this form they do not give rise to further generations.

Discussion

The essentially monodisk character of strobilation just described is much more reminiscent of the strobilae of the rhizostomids *Cotylorhiza tuberculata* (Claus, 1892) and *Cassiopea xamanchu* (Bigelow, 1900) than the polydisk strobilation described for *Cyanea lamarckii* by Delap (1905) at Valencia, and much more extreme than that of *Cyanea arctica* as described by Hargitt (1910) from Woods Hole. The question arises whether the differences indicate different parentage or a varying response to different conditions of growth.

Both size and shape appear to determine the type of strobilation, and since there is the possibility that the type may vary greatly with external conditions, it may be well to exclude Aurelia as a candidate somewhat more definitely. In the first place, a freshly liberated ephyra of Aurelia has a relatively shorter manubrium, gastral filaments much more remote from the manubrial base, and less suggestion of inter-rhopalial tentacles, than the ephyra of our present form shortly before liberation. Secondly, the manner of budding of the scyphistomae is markedly different. If the choice lies between Aurelia and Cyanea, as it appears, there is little doubt that Cyanea is the parent form.

The growth of a scyphistoma up to the time of liberation of an ephyra is divisible into three phases. The first concerns the transformation of the planula into a 16-tentacle scyphistoma. This phase has been intensively studied in relation to the manner of origin of the stomach pouches and the order in which the tentacles arise. Neither of these features greatly concerns us here; our main interest lies in the manner of growth and budding of the scyphistoma, and in the strobilation to form ephyrae.

The second phase, the growth of the 16-tentacle scyphistoma, is associated with the production of buds. In both the rhizostomids, Cotylorhiza and Cassiopea, buds arise one at a time from the scyphistoma body wall above the apex of the stalk. The buds break free, are ciliated and free-swimming, but they eventually settle and become attached by their original outer end.

In the semaeostomids Aurelia and Chrysaora, buds are formed initially as lateral outgrowths from the body wall near the base of the scyphistoma. They grow out as stolons for a considerable distance before becoming attached (Fig. 5B) either to give rise to a new scyphistoma at the point of attachment, or to one or two scyphistomae at some place between origin and attachment. The connection with the parent is finally broken.

In our Cyanea the buds arise from a site equivalent to the point of origin in Cotylorhiza and Cassiopea, but grow longer and downward to become attached basally by the time separation from the parent takes place. In both types, however, the scyphistoma head grows from the upper end of the bud. It is therefore intermediate in character between that of Aurelia and Cassiopea. The three kinds

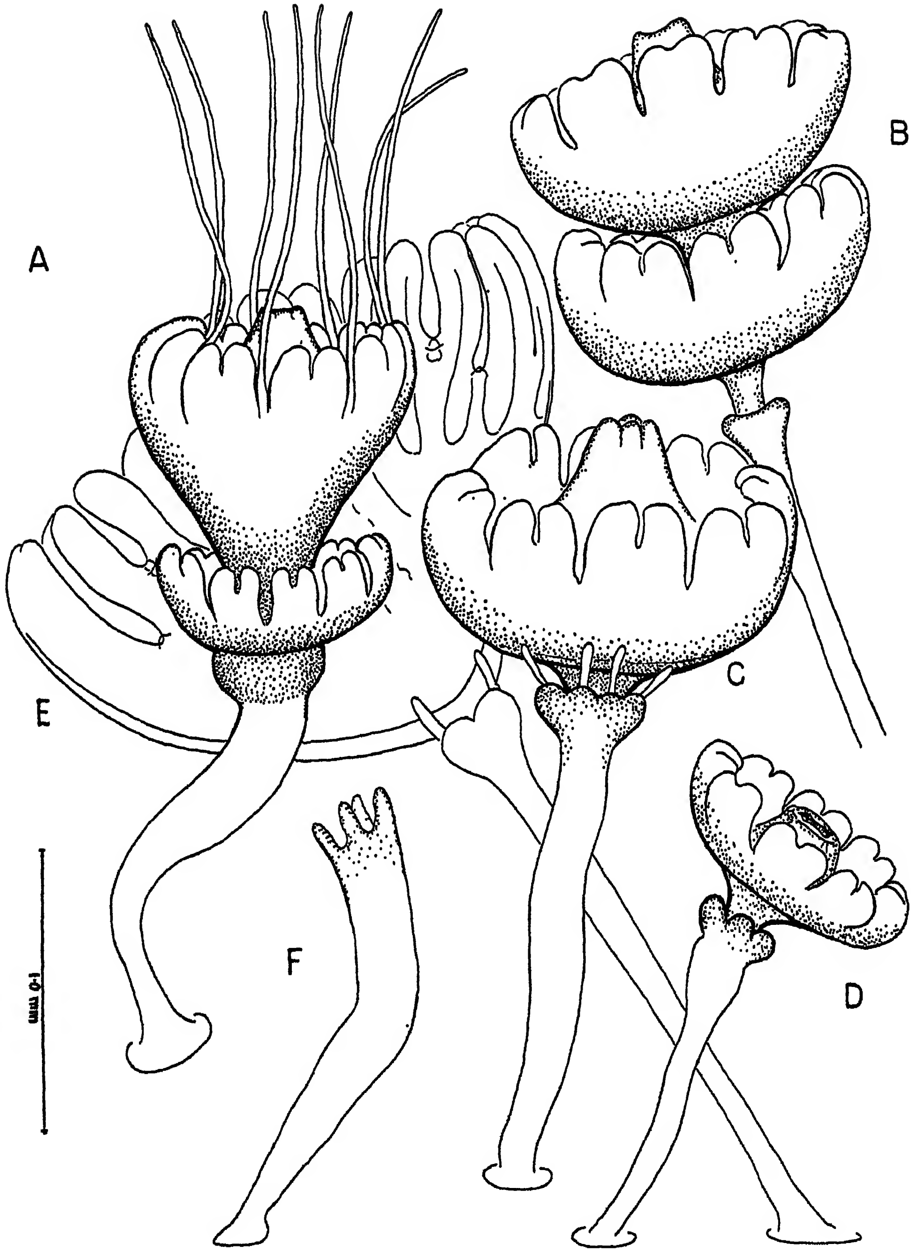

FIGURE 4. Strobilae of *C. capillata*. A, strobila with two ephyra and possible third. B, strobila with two equalized ephyrae. C, strobila with advanced ephyra and a potential second bearing scyphistoma tentacles. D, strobila with second ephyra well developed and a potential third. E, ephyra on point of liberation. F, post-strobila stalk with four tentacles.

of buds are essentially the outcome of two variables, the direction of outgrowth and the intensity of growth. Subsequent development depends upon the orientation of the outgrowth, and a new scyphistoma always arises from an upper surface, whether it be the distal or proximal end of an outgrowth or from some point on its side wall.

The question of monodisk or polydisk strobilation concerns both size and shape, both of which are expressions of growth. In monodisk development, growth in the basal part becomes progressively linear and apparently becomes arrested, while anterior growth becomes progressively transverse. Between the two regions there is a steep growth gradient producing a comparatively abrupt transition from head to stalk.

In contrast to this, the scyphistoma of Aurelia exhibits no such differentiation, and both transverse and linear growth occur throughout, so that while growth in length of the whole is the greater, transverse growth continues in basal as well as anterior regions. A large scyphistoma is therefore not very different in shape from a small one.

Shape is probably one of the main factors in determining the nature of strobilation. Constrictions carve off the shallow saucer-like discs of the scyphistoma to form ephyrae, and whether one, two, or many such discs can be produced is mainly a matter of the shape of the whole and the extent of growth occurring at the various levels. In this light, the difference between monodisk and polydisk strobilation is primarily a difference in the extent to which significant transverse growth can be maintained along the antero-posterior axis of the scyphistoma (cp. Fig. 2A, 5C). This activity may well vary with different conditions of temperature and food supply.

The scyphistomae reared by Delap grew steadily through summer months, apparently without producing buds, in each of two successive years, and in each year strobilated to form eight to eleven ephyra in late winter when the temperature fell below 45° C. The scyphistomae were abundantly fed with small planktonic organisms throughout the whole period. Those reared by Hargitt were fed even more concentratedly, at relatively high temperatures, and grew to the strobila condition with astonishing rapidity. One to five ephyrae were produced, with an average of three to four. Hargitt states that buds were seen but were extremely rare. Fortunately Delap gives the scale of her drawing of the strobila, so that a comparison of actual size is possible. Her polydisk strobilae are approximately three times the height of ours, and have no sharp division into stalk and head.

Our own scyphistomae were without doubt collected during the summer or late spring, and in Passamaquoddy waters would accordingly be developing at low temperatures (below 50° C.), even though maximum for the region. Growth would be relatively slow at the prevailing temperature and the food supply would probably fall far short of the degree of forced feeding employed by Hargitt and Delap.

The form of the sessile phase of the Hydromedusae responds sharply to varying conditions of temperature and food supply (Berrill, 1948, 1949) and it would be expected that the scyphomedusae would also react, in their own way. Differences in relative growth rates, however, may very well be inherited within the limits of a single species, and different races of *Cyanea capillata* may vary in the quantitative growth response their respective scyphistomae make to changing external conditions.

Summary

The developmental cycle of a scyphomedusa, probably *Cyanea capillata* Eschscholtz, is described, with emphasis upon the correlation of size and form.

The nature of the budding process, giving rise either to free buds or to double-headed forms, is described.

An analysis of monodisk and polydisk strobilation is given in terms of growth, size and shape.

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FUNDAMENTAL PRINCIPLES IN OXIDATION–REDUCTION[1, 2]

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Oxidation of organic compounds is the source of energy for living organisms. This mechanism of supply of energy is made possible by the fact that the organic compounds used as food, as well as the oxidizing agent, molecular oxygen, are inert and can, at the proper time, be activated by catalysts so as to interact with each other. This inertia is due to the principle of "compulsory univalent oxidation" or of "single-electron transfer." This may be explained as follows. Oxidation is, primarily, the withdrawal of electrons. It is unessential for the process proper of oxidation whether a proton is withdrawn together with the electron. If so, oxidation is the same as dehydrogenation. Reduction is the reversal of oxidation. The principle just mentioned states that any bivalent (or polyvalent) oxidation or reduction has practically no other chance to proceed than in successive transfers of a single electron, or, in "univalent steps" of oxidations or reductions. These steps may overlap, and often to such an extent that the nature of the two-step process is difficult to recognize. In this way, "bivalent" oxidation or reduction of such substances as quinones and dyestuffs was conceived until recently as a bivalent process occurring by the simultaneous transfer of a pair of electrons.

The experimental evidence for the principle of single-electron transfer can be furnished essentially by two methods: measurement of redox-potentials, and measurement of magnetic susceptibility.

In a reversible redox system, if it is a univalent one, such as Fe^{+++}—Fe^{++}, the molecular species involved can exist on two levels of oxidation. If it is a bivalent one, according to the principle of single-electron transfer, it can exist on three oxidation levels: the reduced form, R; the semioxidized, S; and the totally oxidized, T, which are related to each other as follows (e is the electron):

$$R \rightleftarrows S + e$$

$$S \rightleftarrows T + e$$

The equilibrium $2S \rightleftarrows R + T$ is always established with unmeasurably high speed, just as the equilibrium of electrolytic dissociation, in contrast to most other reactions in organic chemistry which usually are relatively slow, the rate being measurable and strongly dependent on temperature.

All valence-saturated organic compounds have an even number of electrons, each chemical bond being represented by an electron pair. So, any S compound

[1] Paper presented as part of a Symposium of the Society of General Physiologists, Woods Hole, September, 1948.

[2] This paper was originally presented with the aid of about fifty lantern slides representing experimental evidence and a few demonstrations of experiments. The abstract given here is made up in a form supposed to be understandable without this aid.

must have an odd number of electrons; it must be a free radical. Two molecules of a free radical may or may not combine to form a dimer, D, which again has an even number of electrons. In most cases, among organic dyestuffs of the type of methylene blue, or the flavine dyes of the yellow respiration enzymes, the intermediate form is a free radical, S, and not D. The existence of an intermediate form, be it S or D, can be easily recognized on oxidizing R or reducing T, because the intermediate form usually has a color of its own. Whether it is S or D can be recognized as follows. When a solution of R is titrated with an oxidizing agent, and the potential at a blank platinum electrode is plotted against the percentage of oxidation, the curve obtained in this plot is independent of the initial concentration of R, if the intermediate form is S; in contrast, its shape strongly depends on the initial concentration if the intermediate form is D. The experiment shows that in most cases there is S; in some cases, at higher concentraton, some D may be in equilibrium with S. Furthermore, the equilibrium constant, called the semiquinone formation constant

$$\frac{[S]^2}{[R] \times [T]} = k$$

can be measured with this method: The slope of the titration curve depends on k. One can calculate k from this slope. If k is very large (> 16) the titration curve is not simply S-shaped but shows a steepening around 50 per cent oxidation, which directly manifests the two steps of the oxidation.

The magnitude of k depends on the chemical nature of the redox system and on pH. For cationic redox systems (say, basic dyestuffs) k increases with decreasing pH; sometimes the separation into two steps is clearly recognizable in extremely acid solution, e.g. in methylene blue. For anionic redox systems (such as quinone systems), k increases with increasing pH. However, k never becomes vanishingly small for reversible redox systems. The fact that S at any pH is capable of existence in a finite concentration, is the condition *sine qua non* for the reversibility of the redox system. If k is utterly small, it means that the S state is capable of existence only in infinitely small concentration. Since the oxidation has to pass through the S-state, it means that the rate of the oxidation or reduction is slow, that a high activation energy is required both in the direction $R \rightarrow T$ as well as $T \rightarrow R$. For instance, the oxidation of ethanol to acetaldehyde is irreversible and needs a high activation energy because the S form (which would be $CH_3 \cdot \dot{C}H \cdot OH$, a free radical with "tervalent" carbon), is utterly unstable. If this process has to be made reversible, as it is in the living organism, some means must be provided to increase the stability of the S form.

Another method of demonstrating a free radical, S, during the reduction of $T \rightarrow S$ is the measurement of magnetic susceptibility. Since the uncompensated spin of the odd electron in a free radical must produce paramagnetism, free radicals can be recognized, and their concentration determined, by the measurement of magnetic susceptibility. When the solution of a suitable quinone is slowly reduced by glucose in an alkaline solution, the magnetic susceptibility changes first in a direction indicative of the appearance of a free radical, later in a direction to indicate its disappearance again.

Not only does the oxidation of organic compounds as used for food need activation, but also molecular oxygen needs activation for its reduction. The successive steps of the reduction of O_2 are:

$$O_2 \xrightarrow{1} O_2^{-} \xrightarrow{2} O_2^{=} \xrightarrow{3} O_2^{\equiv} \xrightarrow{4} O_2^{≣}$$

or, in presence of water, which can furnish protons:

$$O_2 \xrightarrow{1} O_2H \xrightarrow{2} O_2H_2 \xrightarrow{3} OH + OH_2 \xrightarrow{4} 2OH_2$$

The barrier for this reaction is represented by the high energy content of the radicals O_2H and OH. A high activation energy is required to reduce oxygen to hydrogen peroxide, and also to reduce hydrogen peroxide to water.

Enzymes concerned with oxidation-reduction exhibit their function in lowering the activation energy. The enzyme forms a reversible compound with the substrate. In such enzymes there are always two substrates: the electron acceptor (such as O_2, or Fe^{+++}, or a flavine dye), and the electron donor (such as glucose or lactic acid). One of the two "substrates" represents a coenzyme or a prosthetic group such as heme. It is over and over again reduced and oxidized reversibly in the course of metabolism. The other "substrate" is the substrate proper. Although probably all oxidation-reduction processes are conducted in a reversible manner by enzymes, the whole process, as far as the "substrate" proper is concerned, goes one way only because the reaction product is immediately removed and shuttled on to another enzyme which will cause a further step in its metabolic change.

The problem as to how the enzyme brings about the lowering of the activation energy may be answered as follows. The attractive force exerted by an enzyme to its substrate, resembling that of the force between a protein and its immunological antibody, brings about the enzyme-substrate compound, and even the ternary compound consisting of apoenzyme (the protein part of the enzyme), prosthetic group and substrate proper. The specific shape of the protein surface forces the substrate molecule into a shape not attainable spontaneously. The energy released by the formation of the compound is not entirely dissipated as heat but used to distort the substrate in such a manner as to ease the making of the free radical which is the necessary intermediate step of the oxidation or reduction. The free radical does not exist, under these conditions, in a free state, but as an intramolecular constituent of the enzyme-substrate complex only. The case is comparable to the "activation" of hydrogen by platinum black. The attraction of Pt towards H_2 is strong enough to squeeze the H_2 molecule into the lattice of the Pt atoms in which it does not entirely fit. The H_2 is hereby stretched so that it behaves now almost as though it consisted of two H atoms. An H atom is the analogue of an S form; it contains an odd number of electrons, namely one. The difference is that the enzyme is specific, sometimes for the electron donor, sometimes for the acceptor, and often for both.

PLANT HORMONES, GROWTH AND RESPIRATION [1]

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One of the greatest values of the discovery of the auxins as growth hormones in plants was that they made it possible to control growth. From the study of growth as produced under controlled conditions by auxins came a number of experiments on the interrelation between respiration and growth. Some of these, and the conclusions to which they lead, will be reported in this paper. Problems concerned with the rate of formation, use, and inactivation of auxin in the intact plant represent another field of endeavor which will not be considered herein. It should also be made clear at the outset that the precise chemical nature of the auxin of higher plants need not concern us here. Most of the experiments below were carried out with indole-acetic acid, which is a natural auxin of widespread occurrence in both higher and lower plants. For this type of work the auxin is regarded merely as a tool to produce growth at will.

Now the central problem in regard to auxin and the growth of plants is an old one; namely, how it is that one substance can produce many different kinds of effects. Visible growth in plants, such as stem elongation, is mainly growth by cell enlargement, while the formation of roots or fruits rests in the first stages on a great stimulation of cell division, which only later is followed by enlargement. Yet both these processes are controlled by the supply of auxin to the tissues. The direct effect of auxin on the cambium is also stimulation of cell division. Elsewhere, as in lateral buds, auxin, in physiological concentrations, causes complete inhibition of growth. Such a diversity of the ultimate effects of one hormone suggests strongly that the results observed are remote from the initial action, and that this initial action of auxin on the cell is a fundamental one exerted on some process of metabolism. From this hypothetical change in metabolism the visible effects ensue, according to the age and location of the cell or tissue, the supply of water and of both plastic and catalytic materials, and perhaps also the interaction of auxin with other specific substances.

The purpose of this paper is to consider the evidence that auxin brings about growth through causing a change in metabolism. Now it is known in a general way that growth of higher plants is aerobic and does not take place in nitrogen. This was first shown for a specialized growth reaction, namely geotropic curvature, by van Amejden in 1917. More than ten years ago J. Bonner found (1936) that growth of the oat coleoptile is directly dependent on respiration and is inhibited by cyanide to the same extent that respiration is. On this account van Hulssen tried

[1] Paper presented as part of a Symposium of the Society of General Physiologists, Woods Hole, September, 1948.

[2] I wish to acknowledge the assistance and contributions (many of them unpublished) of Dr. Walter D. Bonner, Jr., and of several students, past and present, including Dr. Schneider, Dr. Commoner, Dr. Sweeney, and Mr. Christiansen.

to detect an influence of auxin on respiration, using oat coleoptiles, but found no effect. Others also obtained negative results, although subsequently it has been found that there are some conditions under which auxin may produce an increased respiration. The absence of any necessary increase in respiration to accompany the increase in growth rate, however, indicates that growth by cell enlargement does not involve any considerable overall expenditure of energy. This has been shown in another way by the calculations of Goddard (1948) and of Frey-Wyssling (1948) whose figures indicate that the actual energy involved in growth is probably not over one per cent of the total energy available to the cell from respiration.

Cyanide, of course, acts on the terminal oxidase and thus inhibits the respiration of all kinds of metabolites. Its effect, therefore, is not specific. It was thought that a fresh approach might be made through studying the effects on growth of the somewhat more specific dehydrogenase inhibitors. This at once proved to be fruitful and has led to numerous metabolic experiments with growth inhibitors.

The Experimental Material

At this point mention should be made of the experimental objects often used in growth studies. The requirement for strictly uniform plants available in large numbers makes it essential to use seedlings, and, because light influences the production of auxin and also causes curvature and other complications, the seedlings are almost invariably grown in the dark, and worked on only in red light. Of such dark-grown (etiolated) seedlings the most widely used is the oat, the coleoptile of which completes its growth in about five days at 25° C., and in which all cell division ceases after the first 10 mm. is reached. This provides an ideal experimental object, in which the growth involves only cell elongation. Sections cut from such coleoptiles grow well in simple auxin solutions, and much better if sucrose is added. The optimum concentrations are about 1 per cent sucrose and 1 to 5 mg. per liter of indole-acetic acid ($= 0.6$ to 3.0×10^{-5} M). Another very satisfactory etiolated seedling is that of the pea; we use sections cut from the apex of the third internode. The growth of these sections is small (about 50 per cent of their initial length) but very reproducible; it is not appreciably affected by the addition of sugar, and hence this material is convenient for chemical studies. The same internodes when slit lengthwise give a large curvature in auxin solutions which has been extensively used in assaying synthetic substances for their auxin activity; this response has the advantage of not being nearly so limited in its applicability as that of the well known "Avena test" of Went, in which the auxin is applied in agar to one side of the decapitated coleoptile.

The growth of seedling sections in pure auxin solution—with sucrose added in the case of the oat coleoptile—is of course a highly limited growth process. Shorn of the complications due to cell division, mineral nutrition, light and nitrogen supply, these sections represent about the simplest system which can still be regarded as growing. The detailed analysis of such a simplified system should, however, be the first step towards an understanding of the whole complex of growth reactions which takes place under natural conditions.

The growth of coleoptile sections is highly aerobic. Even submergence beneath 1 mm. depth of solution retards it about 50 per cent; aeration of such lightly submerged sections restores the rate to its full value (Thimann and Bonner, 1948). In most of our experiments the sections are arranged so as just to break surface.

Growth Inhibition by Iodoacetate

The growth is reduced or prevented by inhibitors of dehydrogenases. The effect of iodoacetate was studied in detail by Commoner and Thimann (1941), who showed that growth of coleoptile sections is strongly inhibited by this substance. However, concentrations of the inhibitor which reduce growth practically to zero have only a very small effect on respiration. (The data on respiration are in excellent agreement with the later measurements of J. Bonner, 1948). Here again it follows that of the total energy released by respiration only a few per cent can be needed for growth, for otherwise the complete inhibition of growth could be achieved only with a substantial inhibition of respiration. This confirms the experiments and calculations mentioned above; most of the energy of metabolism evidently goes for maintenance. However, it is not excluded that growth might involve an appreciable fraction of the respiration, but that when this process is inhibited another type of respiration might take its place, so that the total oxygen consumption would show little change. We shall return to this important consideration below. It is also necessary to point out that in the pea stem the relations are not quite the same, for here auxin does produce a slight increase of oxygen consumption, and growth inhibition is accompanied by a definite respiration decrease (see below).

An interesting and important effect was observed with iodoacetate; the sensitivity to this inhibitor varies with the age of the coleoptile. This is shown in Figure 1. Young coleoptiles show an incomplete inhibition, as well as a marked

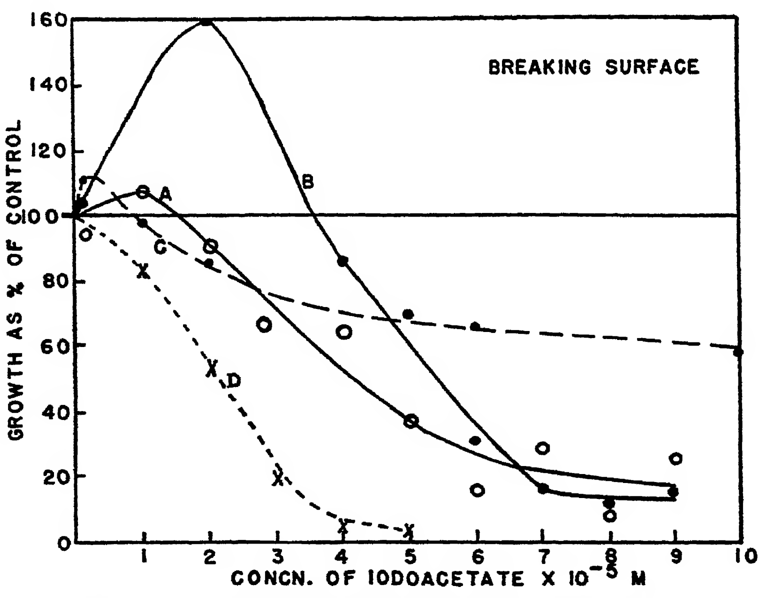

Figure 1.[3] The total growth, after 48 hours, as per cent of that of the controls, plotted as a function of iodoacetate concentration. Curve A, sections from 74 hr. coleoptiles; curve B, from 64–66 hr. coleoptiles; curve C, from 54–56 hr. coleoptiles; and curve D, from 96 hr. coleoptiles. All solutions contained 1 mg. indole-acetic acid and 10 grams sucrose per liter.

[3] Figures 1, 2, 3, and 5 are from Thimann and Bonner (1948 and 1949).

acceleration at low inhibitor concentrations; with the oldest coleoptiles, on the other hand, the concentrations necessary for threshold and for 50 per cent inhibition are much lower, and the maximum inhibition is very high. Two explanations are possible for this effect:

(a) the amount of enzyme with which the iodoacetate has to combine decreases with increasing age;
(b) the young plant contains substances which oppose the inhibition and which decrease in amount with increasing age.

Evidence for the latter view will be presented below. First, however, it will be convenient to consider in more detail the nature of the enzyme system.

Sulfhydryl Nature of the "Growth Enzyme"

It is known that iodoacetate (or iodoacetamide, which behaves similarly) reacts with sulfhydryl groups, although as Michaelis and Schubert have shown (1934) it is not strictly specific for these. Since the enzyme system which controls growth is of great importance, it seemed worth while to determine definitely whether it is of sulfhydryl nature or not. The action of a number of other inhibitors has given clear-cut evidence on this (Thimann and Bonner, 1949).

Arsenite and the organic arsenical mapharsen inhibit growth strongly. The effective concentrations are lower than for iodoacetate. The extent of inhibition by arsenite, unlike that by iodoacetate, does not vary with the age of the coleoptile (Fig. 2). The growth of pea stems is also inhibited by arsenite, showing exactly

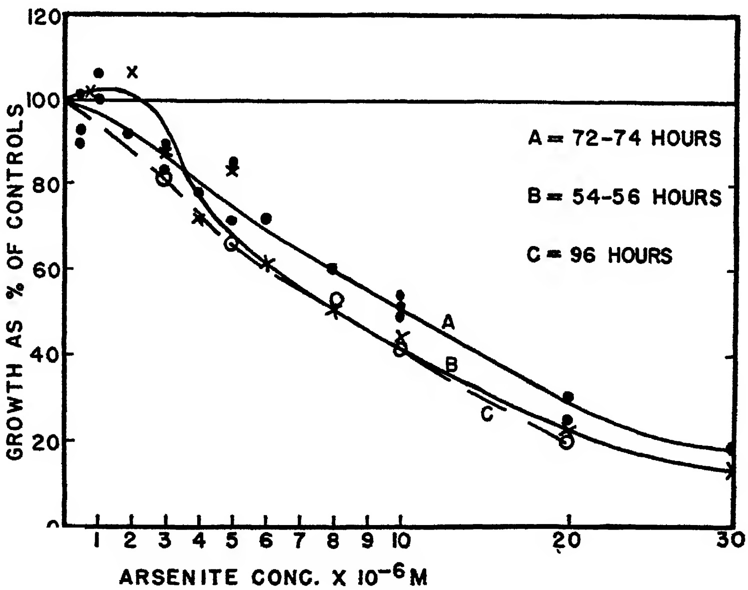

Figure 2. Data similar to Figure 1 but for arsenite. Sections from the three ages of coleoptiles show no significant difference in sensitivity to the inhibitor.

the relationship with arsenite concentration to be expected of a titration curve (Fig. 3). Such inhibitions can, of course, be regarded as titration of the enzyme with the inhibitor. The "titration curves" of iodoacetate are complicated by the promotion of growth at low iodoacetate concentrations; arsenite does not produce this effect.

A more specific sulfhydryl reagent is parachloromercuribenzoate, introduced by Hellerman et al. (1943). This also inhibits growth and, like arsenite, the effective concentrations do not vary appreciably with age. Again the growth and slit stem curvatures of peas are also inhibited by this reagent.

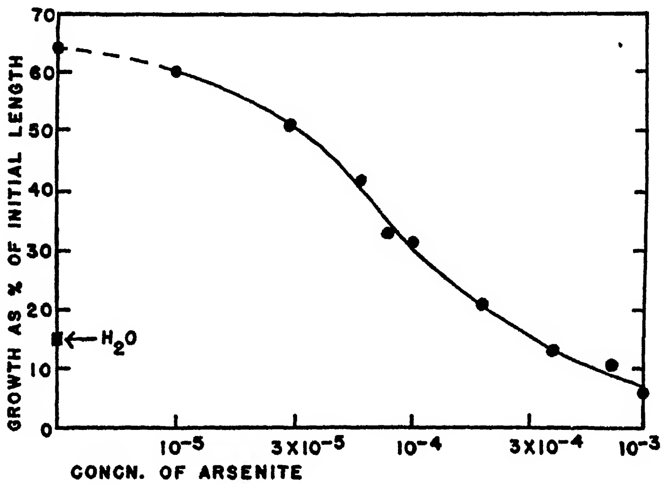

FIGURE 3. Growth, after 24 hrs., of 20 mm. sections of the uppermost internode of 7-day-old etiolated pea stems. All solutions contained indole-acetic acid 10 mg. per liter and arsenite as shown, but no sucrose. Growth of controls in water is shown at left.

Finally the phenyl-mercuric salts inhibit growth strongly. The concentration for 50 per cent growth inhibition of coleoptile sections is very low, about 7×10^{-6} M. However, there is reason to believe that these substances are not so specific as those above, since they definitely inhibit respiration. At concentrations which produce 50 per cent inhibition of growth, arsenite, iodoacetate and parachloromercuribenzoate exert no detectable effect on coleoptile respiration, as shown in Figure 4. The phenyl-mercuric salts thus inhibit somewhat in the same way as cyanide.

Taking the data together it is clear that the growth-controlling enzyme is of sulfhydryl composition. This conclusion holds far beyond the higher plants on which this work was done, for long ago Hammett, Voegtlin, Chalkley and others adduced evidence for the importance of the SH-group in the growth of invertebrates, and recently, Ryan, Tatum and Giese (1944) showed that the growth of Neurospora is inhibited by iodoacetate in a manner both quantitatively and qualitatively similar to that of coleoptile sections. This clearly is an aspect of General Physiology.

The Role of Organic Acids in Growth

Some years ago Commoner and Thimann (1941) found that the inhibition by iodoacetate is prevented by malate, succinate, fumarate and pyruvate. More recently we have confirmed this and refined the technique of such experiments. Isocitrate has a similar effect; so also, unexpectedly, do maleate and malonate. The growth system behaves in this respect like succinic dehydrogenase, which has been shown by Hopkins, Morgan and Lutwak-Mann (1938) to be "protected" against iodoacetate by these acids. However, while maleic and malonic acids bring the growth rate in presence of iodoacetate back to normal, they have no further effect; malate, succinate, fumarate and isocitrate bring the growth rate considerably above normal (Fig. 5). In other words these acids accelerate growth. There are thus two phenomena to be distinguished: protection against iodoacetate, and promotion of growth. Maleate and malonate, which protect the SH group against iodoacetate, do not promote growth, and indeed in higher concentrations (0.05 M) actually inhibit it. Growth promotion is limited to the acids mentioned above, with the

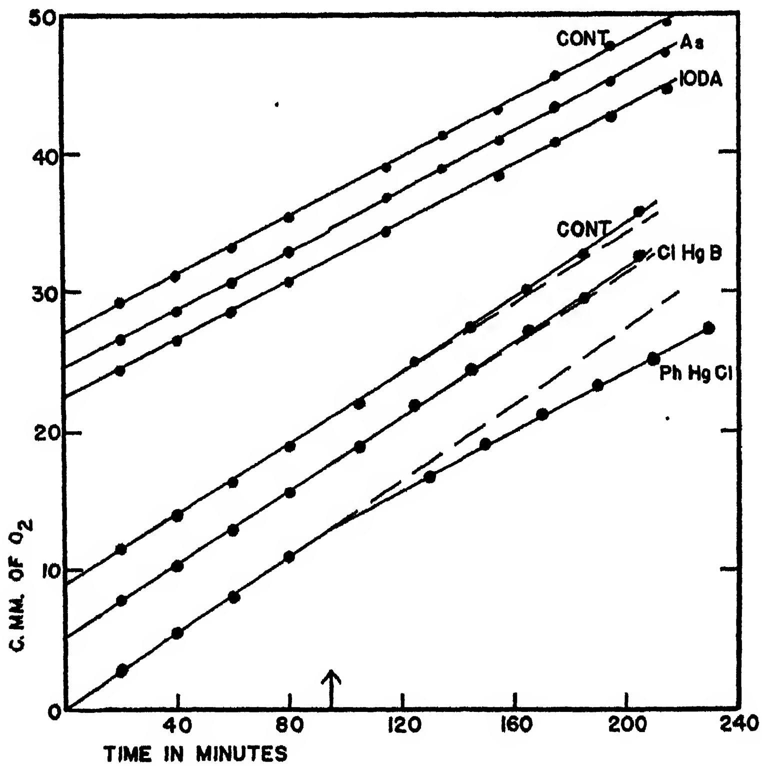

Figure 4. The respiration of sections from 72-hour-old coleoptiles in indole-acetic acid 1 mg., sucrose 10 gms., per liter. Two experiments are shown; the uppermost curve being the controls in each case. The inhibitors were added at the arrow, in concentrations which cause 50 per cent decrease in growth. As: arsenite 1.10^{-5} M As_2O_3. IODA: 5.10^{-5} M iodoacetamide. ClHgB: 4.10^{-5} M parachloromercuribenzoate. PhHgCl: 1.10^{-5} M phenyl-mercuric chloride.

ddition of pyruvate (which is very active when pure), of citrate for young plants nly, and of acetate.

These acids active in growth are those of the Krebs cycle. The importance f this cycle in respiration is well known, and recently J. Bonner (1948) has made . probable that the same or a similar cycle occurs in the respiration of coleoptile issue. The experiments above, and several others, make it clear that malate and ther acids of the cycle actually participate in the normal growth process.

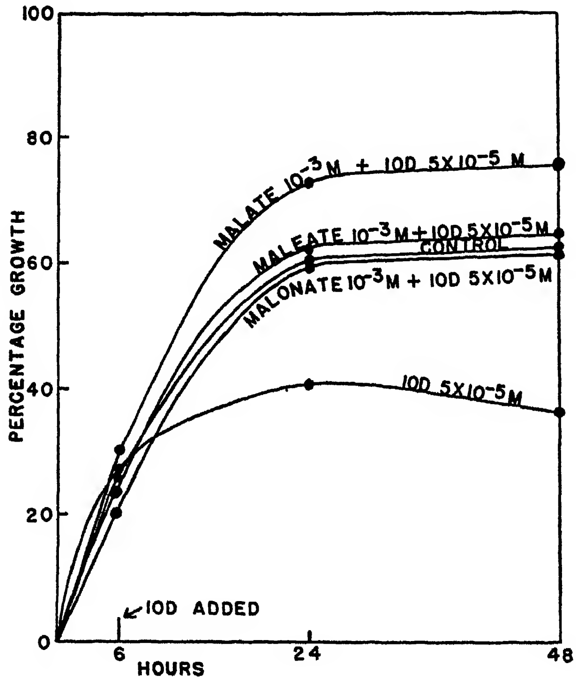

FIGURE 5. The effects of malonate, maleate and malate in preventing the iodoacetate inhibition of growth. Sections cut from 66-hour-old coleoptiles, breaking surface, in solutions containing 1 mg. indole-acetic acid and 10 grams sucrose per liter. The iodoacetate was added 7 hrs. after placing the sections in organic acids. While all three acids act against iodoacetate, only the malate increases growth above that of the controls.

For one thing, treatment with malate makes possible a direct demonstration that auxin does influence respiration in the coleoptile. For if coleoptile sections, which show no increase in respiration when auxin is added, are first soaked for some hours in malate or fumarate, then the addition of auxin at once increases their respiration rate. This means, of course, that malate (etc.) is required for auxin to exert its full effect, and that when this effect is exerted on growth it is by way of a respiratory system.

The role of the organic acids in facilitating the action of auxin also makes possible an explanation of the effect of age on growth. Some years ago, Mrs. Sweeney and I (1942) made a study of the effect of auxin on the rate of protoplasmic streaming in the epidermal cells of the coleoptile. The usual effect of

physiological concentrations of indole-acetic acid or other auxins is to accelerate the rate of streaming; the process requires both oxygen and sugar, and from its dependence on auxin concentration and other features, we deduced that it is closely related to the promotion of growth. Now when the coleoptiles are very old (120 hours) this acceleration of streaming no longer occurs, but it was found that if the old coleoptiles are soaked in malate for some hours, the acceleration is reinstated. This would suggest that in old coleoptiles the concentration of malate has greatly decreased. *A priori* this would seem unlikely since it is known from experiments on the excystment of protozoa that in fully grown grasses the opposite is the case—organic acids increase markedly with increasing age. However, W. Bonner has recently shown by direct microanalysis that the amount of malate and other organic acids in the coleoptile does decrease with increasing age (Table I).

TABLE I

Organic acids of Avena coleoptiles and pea stems
All figures per gram dry wt.

AVENA SECTIONS	TOTAL ETHER-SOLUBLE ACIDS micro-equivalents	CITRIC mg.	MALIC mg.
54 hours	432	1.44	13.7
72 hours	329	1.05	10.2
96 hours	284	1.02	5.7
PISUM STEMS	616	1.29	13.7

Another interesting point shown in the table is that the pea stems contain more organic acids than any age of coleoptile. Now the pea stems do not show increased growth with malate. Furthermore, they differ from the coleoptiles in another important respect: the addition of auxin causes a direct increase in respiration rate. This increase is about 15 per cent and is maintained for 24 hours or throughout the whole period during which growth of the sections takes place. In other words, the pea stems behave like coleoptiles pretreated with malate. The correlation between this behavior and their content of malate and other acids shows how the age effect in coleoptiles is to be interpreted; the increasing sensitivity to iodoacetate with increasing age is due to a decreasing content of the organic acids, which "protect" against iodoacetate. Correspondingly, aging causes no increase in sensitivity to arsenite or parachloromercuribenzoate, because the organic acids do not protect against these reagents.

There are other substances whose decreasing concentrations play a role in growth. Preliminary determinations of the keto-acids indicate that they behave in a similar way. J. Bonner (private communication) has recently shown that arginine also plays a role in growth of coleoptiles, which can be accelerated by adding arginine and inhibited by adding the related substance canavanine. In our experiments the promotion of growth by arginine is limited to older coleoptiles, so that the amount of this substance appears to decrease with age, like the organic acids. Doubtless still other materials behave similarly. However, to be able to

explain even one aspect of aging in terms of a decrease in concentration of known substances in the plant is a definite step forward.

Why should the amount of these substrates decrease with age? To some extent the reason might be merely that the amount contributed by the seed is limited, so that the substances are essentially diluted by the increasing volume of the seedling. But in the case of some of the organic acids there is evidence, which will not be presented here, that they are actually used up in the growth process, and when growth is inhibited, the rate of their disappearance slows down. This brings us to the last part of the subject, namely the phenomena occurring during inhibition.

Metabolic Changes Associated With Growth Inhibitors

Since the pea stem grows very well in auxin solution without sucrose, and shows little increase in growth when sugar is added, it seemed to us excellent material for a study of the fate of carbohydrates during growth and inhibition. Several conclusions can be drawn from this work. It appears, first, that the amount of reducing sugar which disappears during growth is exactly the same in auxin as in water. When it is considered that the growth in auxin is three or four times that in water, and that the R.Q. in both cases is close to 1, this result is surprising. Changes in non-reducing sugars are small and only minute amounts of metabolizable polysaccharides are present. It follows that the consumption of sugar in growth, both as a metabolic substrate and as a constituent of cell-walls, is negligibly small.

Secondly, when growth is inhibited, reducing sugars do not pile up as might be expected, but instead their amount decreases. With 50 per cent growth inhibition by iodoacetate, the reducing sugar decreases about 25 per cent more than when growing without inhibitor. The same effect is shown by other inhibitors. Typical results are shown in Table II (Christiansen et al, 1949). Fluoride has not been mentioned above as an inhibitor; its effect in lowering the reducing sugar is somewhat greater than that of the sulfhydryl-combining inhibitors. This, however, may be due to the fact that it slightly increases respiration after some hours, instead of decreasing it as do the others.

Naturally the fate of the reducing sugar which disappears when growth is inhibited is of the greatest interest. It is not respired away, since respiration is (except with fluoride) decreased. It is not converted to detectable amounts of starch, and chemical analyses show that it is not deposited as cellulose or any other wall materials. It is not converted to phosphate esters either, since the amounts of these, both in inhibited and uninhibited sections, are very small in comparison to the amounts of sugar involved.

In brief it appears that, though the effect is qualitatively the same with different inhibitors, the ultimate fate of the sugar differs in each case. In arsenite the sugar is converted to neutral ether-soluble material, i.e. fats. In fluoride the same conversion occurs but some of the material is respired away, while in iodoacetate, surprisingly enough, it is excreted into the solution in the form of organic acids, or, more strictly, a quantity of organic acid equal in weight to the sugar which has disappeared is excreted into the solution. It should be noted that growing tissues normally accumulate solutes vigorously from solution so that an excretion (or exosmosis) in quantity is most unusual. The nature of this stem exudate is now under investigation; it contains some fructose and 15 per cent of asparagine.

In general the experiments show that inhibitors not only inhibit one process (which leads to growth), but they also promote another, which consumes sugars. In this respect the phenomena are suggestively similar to the "uncoupling" of phosphorylation by dinitrophenol (Loomis and Lipmann, 1948) which also leads to increased disappearance of sugar. Indeed dinitrophenol does inhibit growth, though we have not yet studied it in detail. Space does not permit the detailed presentation of data on other inhibitors but it may be mentioned (a) that fluoroacetate, which specifically inhibits acetate metabolism, causes a partial growth inhibition which can be reversed by adding acetate, and (b) that poisoning the enzyme which decarboxylates pyruvic acid does not inhibit, but actually promotes growth.

TABLE II

Growth, respiration and reducing sugar of 20 mm. sections of etiolated pea stems after 24 hrs. in solution

	GROWTH as per cent elongation	REDUCING SUGAR as per cent of fresh wt.	RESPIRATION as Q_{O_2} (after 12 hrs.)	PER CENT DECREASE		
				of growth in length	of reducing sugar	of respiration
Initial	—	1.12	—	—	—	—
In water	20.0	0.83	3.34	—	—	—
In auxin (1 mg./l.)	50.9	0.86	3.82	0	0	—
In auxin plus:						
Iodoacetate 6×10^{-4} M	25.6	0.64	3.32	50	26	13
1×10^{-3} M	18.2	0.54	—	64	37	—
Arsenite 1×10^{-4} M	26.5	0.71	2.89	48	17	24
1×10^{-3} M	0	0.51	—	100	41	—
Fluoride 5×10^{-3} M	25.3	0.48	4.47	50	44	17
1×10^{-2} M	17.8	0.42	—	65	51	—

The relation between respiration and growth is thus not a simple one. The process mediated by the SH-enzyme appears to be a major limiting factor. It either consumes only a small part of the total oxygen and sugar used, or else it is readily replaced by an equivalent reaction not causing growth, and it probably is a step in the oxidation of pyruvic acid via the Krebs cycle or some modification of it. The SH-enzyme is almost certainly one of the dehydrogenases of this cycle. It may be noted in passing that attempts to demonstrate an iodoactetate-inhibited enzyme of this type in coleoptile brei, by Berger and Avery (1944), failed, but this would be expected from the data presented above, because the test has to be

made in presence of malate or other dicarboxylic acid, and these very substrates protect the enzyme fully against the inhibition by iodoacetate. It also appears that the simple decarboxylation of pyruvic acid does not lead to growth, but its oxidative decarboxylation and the resulting metabolism of acetate does do so. The inhibition of growth in presence of auxin is not the same as the mere absence of growth in sections not supplied with auxin; it is a positive process and leads to the diversion of sugar metabolism into other pathways. The action of an inhibitor is that of a switch, diverting the stream of traffic into a direction which does not lead to growth. Correspondingly the action of auxin itself may also be that of a switch, causing the metabolism of carbohydrate, or perhaps more specifically that of pyruvate, to go via the growth-promoting system instead of by another. It may be, therefore, that the apparently small fraction of metabolism involved in growth is a misleading phenomenon and that the true picture is rather one of alternative routes involving approximately equivalent amounts of respiration. However, such considerations are at present only speculative.

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THE

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THE MARINE BIOLOGICAL LABORATORY

FIFTY-FIRST REPORT, FOR THE YEAR 1948—SIXTY-FIRST YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time

and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 10 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years; and in addition there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. THE REPORT OF THE TREASURER

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen:

The year 1948 from a financial point of view was quite satisfactory. Increased use of the Laboratory's facilities and continued progress in the program of repairs and additions to buildings and equipment were reflected in considerably increased expenditures. The total paid out for current expenses was $264,897.87. The amount spent for additions to capital assets, including $22,856.84 paid on the new boats, was $53,814.81, so that total expenditures were $318,712.68.

Fortunately, current receipts also increased to a total of $291,044.89. To this sum was added $26,539.78 in special, non-recurring receipts that made a total of $317,584.67, so that on a cash basis, the deficit for the year was only $1,128.01. These special receipts consisted of $4,708.11 from the sale of securities given in recent years by Mrs. W. Murray Crane for current expenses and applied with her permission towards the purchase of the "Dolphin," $6,218.88 balance remaining in the Reserve Fund, $9,300.00 given by the American Cancer Society and used chiefly for new equipment, and $6,312.79 balance remaining in the Boat Fund contributed in 1947 and 1948.

The year was also notable for the receipt of 500 shares of Crane Company common stock, valued at $17,250.00 from the Estate of Dr. Frank R. Lillie, and the grant from the Rockefeller Foundation of $250,000., $100,000. for current expenses over a period of five years, and $150,000. for the restoration of "Old Main."

As in recent years, the accounts have been audited by Seamans, Stetson & Tuttle, certified public accountants of Boston, and a copy of their report is available for inspection. The "Balance Sheet" as prepared by them is appended as Exhibit A. As of December 31, 1948, the total *book* value of all the Endowment Assets was $979,865.18, an increase for the year of $1,188.51. The *market* value of these assets was $988,639.62, a reduction for the year of $1,174.54. Plant Assets (Land, Buildings and Equipment) amounted to $1,133,168.64, an increase of $21,217.09.

Following last year's precedent, the "Statements" which follow, prepared with the assistance of Mr. Homer P. Smith, Assistant Business Manager, give the actual financial transactions of the year on a cash basis excluding accruals, non-cash items, depreciation, interdepartment charges, etc. They are:

I. Summary Cash Statement for Year,

II. Current Expenses by Departments,

III. Additions to Capital Assets from Current Funds,

IV. Retirement Fund.

V. Fellowship Fund,

VI. Special Funds,

VII. Real Estate Accounts,

VIII. Agency Accounts.

I. *Summary Cash Statement for Year Ended December 31, 1948*

	Receipts	Expenditures: Current	Expenditures: Additions to Capital Assets	Expenditures: Total Expenditures
Membership Dues	$ 2,268.00			
Donations for Current Expenses[1]	1,815.00			
Income from Endowment	40,707.09			
Income from Other Securities	23,768.60			
Real Estate Rentals	6,360.00	$ 986.20		$ 986.20
Instruction	12,403.64	8,357.17		8,357.17
Research (incl. Apparatus and Chemical Dep'ts)	25,673.04	26,307.31	$11,346.18	37,653.49
Mess	38,858.06	39,375.21	417.13	39,792.34
Dormitories and Apt. House	16,788.30	14,175.34	327.71	14,503.05
Library[2]	5,989.85	9,431.37	7,983.41	17,414.78
Buildings and Grounds		42,276.09	4,367.57	46,643.66
Supply Department[3]	106,724.12	85,693.13	29,372.81	115,065.94
"Biological Bulletin"	7,538.15	8,345.89		8,345.89
Administration		27,652.96		27,652.96
Miscellaneous	2,151.04	2,297.20		2,297.20
	$291,044.89	$264,897.87	$53,814.81	$318,712.68

Special Receipts

Sale of Securities	$4,708.11	
Reserve Fund	6,218.88	
American Cancer Soc.	9,300.00	
Boat Fund	6,312.79	26,539.78
		$317,584.67

Total Expenditures	$318,712.68
Total Receipts	317,584.67
Cash Deficit for Year	$ 1,128.01

Notes:

[1] Donations were $1,320. given by the "Associates" of the Laboratory and $495. miscellaneous contributions.

[2] The Library income consists of $3,000. payment from the Oceanographic Institution towards Library expenses and $2,989.85 from the Carnegie Book Fund (balance of Book Fund still available is $11,416.15). The monetary value of serials received in exchange for the "Bulletin," estimated at $3,571.26, is not included, nor is the $800. received from the Oceanographic Institution for the purchase of books for their account.

[3] The actual 1948 sales of the Supply Department were $94,701.77, a slight decrease from the 1947 sales. The values of specimens and supplies furnished the Research and Instruction Departments were $8,817.50 and $5,488.82 respectively. If these values are taken into account, and also the gain in inventory of $6,042.36, the decrease in accounts receivable of $14,101.28, and a debit charge of $2,100. for administration and maintenance expense, there would be a net profit of $25,178.39 on the operations of the Supply Department for 1948. This does not take into account the $6,515.97 spent for capital items, exclusive of the new boats, or the auditors' charges of $1,684.38 for depreciation. If these had been included, the net profit for the Supply Department would have been $16,978.04.

II. Current Expenses for 1948 by Departments

Administration

Salaries	$ 21,661.02
Central Hanover Bank Trustee Commissions	1,036.22
Falmouth Bank Charges	154.53
Audit	1,162.35
Treasurer's Office	600.00
Advertising	374.52
Office Supplies	1,320.08
Sundries (Tel., Postage, etc.)	1,803.82
	28,112.54
Deduct Cash Receipts	459.58
	27,652.96

Instruction

Salaries and Travel	8,210.40
Sundries	146.77
	8,357.17

Research (incl. Apparatus and Chemical Dep'ts)

Salaries	14,316.12
Travel	200.00
Repairs	344.66
Supplies and Sundries	13,245.80
	28,106.58
Deduct Cash Receipts	1,799.27
	26,307.31

Library

Salaries	8,947.57
Office Supplies	190.08
Sundries	333.38
	9,471.03
Deduct Cash Receipts	39.66
	9,431.37

Buildings and Grounds

Salaries and Wages	25,253.60
Fuel	3,711.08
Gas	1,557.45
Light and Power	3,320.66
Water	722.29
Insurance	1,706.71
Repairs	4,329.09
Sundries	2,043.13
	42,644.01
Deduct Cash Receipts	367.92
	42,276.09

Dormitories and Apt. House

Salaries and Wages	5,576 27
Lighting, Gas and Water	2,114.74
Repairs	3,082.81
Outside Rentals	500.00
Laundry	1,455.41
Insurance	762.22
Sundries	716.17
	14,207.62
Deduct Cash Receipts	32.28
	$ 14,175.34

Mess

Salaries and Wages	$ 8,800.84
Cost of Food	25,989.86
Gas, Water, Light and Power	2,151.89
Repairs	89.48
Replacements of Dishes, etc.	647.78
Insurance	623.98
Laundry	327.23
Freight and Express	58.08
Sundries	780.16
	39,469.30
Deduct Cash Receipts	94.09
	39,375.21

Supply Department

Salaries and Wages	33,607.64
Purchase of Specimens	32,529.14
Chemicals	2,384.83
Containers	3,826.05
Boat Expenses	2,631.38
Truck Expenses	559.94
Freight and Express	3,107.31
Fuel, Light and Power	809.35
Office Supplies	460.08
Telephone and Telegraph	206.29
Insurance (incl. Boats)	2,381.84
Advertising	392.76
Specimens and Supplies purchased for Research Dep't	1,035.64
Nets, Floats, etc.	461.00
Repairs and Sundries	1,349.08
	85,742.33
Deduct Cash Receipts	49.20
	85,693.13

"Biological Bulletin"

Salaries	2,130.00
Printing, etc.	6,222.84
	8,352.84
Deduct Cash Receipts	6.95
	8,345.89

Real Estate (Leased Out)

Taxes and Insurance on Bar Neck Property (Garage) and Janitor's House	986.20

Other Expenses

Workmen's Compensation Insurance	1,004.67
Truck Expense	462.99
Bay Shore and Great Cedar Swamp Expenses	256.47
Interest on Mortgage	406.67
Evening Lectures	166.40
	2,297.20
Total Expenses	$264,897.87

III. *Additions to Capital Assets from Current Funds*

A. Buildings

Brick Laboratory	$ 2,247.59
Supply Dep't	6,318.12
Mess	202.27
	$ 8,767.98

B. Library

Back Sets	$ 1,903.37
Books	569.15
Serials	2,989.39
Reprints	13.75
Binding	2,507.75
	$ 7,983.41

C. Equipment

Apparatus Dep't	$ 9,069.71
Dormitories	327.71
Mess	214.86
Brick Laboratory	306.97
Carpenter Shop	421.42
Boats	22,856.84
Supply Dep't	197.85
Truck	1,391.59
Machine Shop	2,276.47
	$37,063.42
Total Additions to Capital Assets	$53,814.81

IV. *Retirement Fund* (Securities and Cash)

Jan. 1, 1948, Balance on Hand		$16,388.44
Receipts:		
Income and Principal Gains	$ 243.02	
Payment from M.B.L. (10% of 1948 Payroll)	7,323.62	7,566.64
		23,955.08
Disbursements:		
Pensions paid in 1948	5,710.00	
Custodian Fees	50.00	5,760.00
Dec. 31, 1948, Balance on Hand		$18,195.08

V. Fellowship Fund

Jan. 1, 1948, Balance on Hand		$ 1,043.03
Receipts:		
Payment from Lalor Foundation	$ 5,000.00	5,000.00
		6,043.03
Disbursements:		
Fellowships (nine)	3,665.35	
Laboratory Space, Apparatus and Supplies	1,499.53	5,164.88
Dec. 31, 1948, Balance on Hand		$ 878.15

VI. Special Funds

A. Boat Fund

Jan. 1, 1948, Balance on Hand		$ 5,302.79
Receipts:		
Contributions	$ 1,010.00	1,010.00
		6,312.79
Disbursements:		
Transfer to M.B.L. Income Account for New Boats		6,312.79
		–0–

B. Dr. Frank R. Lillie Memorial Fund

Jan. 1, 1948, Balance on Hand (Initial Contribution from Dr. G. H. A. Clowes)	$ 1,000.02
No Transactions in 1948	

VII. Real Estate Accounts

A. Devil's Lane Property

Cash Received in 1948 from Sales	$ 4,097.50
Disbursements (Taxes)	193.30
Book Value of Unsold Lots, Dec. 31, 1948	36,739.43
Accounts Receivable, Dec. 31, 1948	6,662.00

B. Gansett Property

Cash Received in 1948	$ 845.00
Disbursements (Taxes)	85.44

All Gansett lots having been sold, cash balance of $522.20 representing net profit on sale of last five lots was transferred to Current Income account, and Gansett account was closed.

VIII. Agency Accounts

A. Cancer Research Account

(U. S. Public Health Service Project under direction of Dr. Robert Chambers)

Jan. 1, 1948, Balance on Hand		$18,721.19
Disbursements:		
Payments for .Salaries, Laboratory Space, Apparatus and Supplies	$18,719.96	
Balance Remitted to the United States Treasury	1.23	18,721.19
		–0–

EXHIBIT A

Marine Biological Laboratory Balance Sheet, Dec. 31, 1948

(From Auditors' Report)

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee		$ 961,729.66	
Securities and Cash in Minor Funds		18,135.52	$ 979,865.18
Plant Assets:			
Land	$ 110,626.38		
Buildings	1,345,956.86		
Equipment	238,252.31		
Library	374,880.39		
	$2,069,715.94		
Less Reserve for Depreciation	747,963.45	$1,321,752.49	
Book Fund, Securities and Cash		11,416.15	$1,333,168.64
Current Assets:			
Cash		$ 15,257.21	
Mortgage Note Receivable		2,350.00	
Accounts Receivable		24,420.03	
Inventories:			
Supply Department	$ 49,974.46		
"Biological Bulletin"	16,195.99	66,170.45	
Investments:			
Devil's Lane Property	43,401.43		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Securities	35,020.00		
Retirement Fund	18,195.08	109,316.51	
Prepaid Insurance		5,959.57	
Items in Suspense (Debits)		2,092.72	$ 225,566.49
			$2,538,600.31

Liabilities

Endowment Funds:			
Endowment Funds	$ 960,016.27		
Reserve for Amortization	1,713.39	$ 961,729.66	
Minor Funds		18,135.52	$ 979,865.18
Plant Funds:			
Mortgage Notes Payable		$ 10,000.00	
Donations and Gifts	$1,172,564.04		
Other Investments in Plant from Gifts and Current Funds	150,604.60	1,323,168.64	$1,333,168.64
Current Liabilities and Surplus:			
Accounts Payable		$ 13,552.25	
Items in Suspense (Credits)		2,582.07	
Current Surplus		209,432.17	$ 225,566.49
			$2,538,600.31

Respectfully submitted,

Donald M. Brodie,
Treasurer

V. REPORT OF THE ACTING LIBRARIAN

1948

In 1948 the Library staff carried on the necessary routine activities and continued with projects already in progress.

The sum of $11,500 was appropriated to the Library, plus $3000 from the Woods Hole Oceanographic Institution. a contribution toward the salaries of the staff. For a detailed account of the expenditures of the budget, $13,077.73, reference may be made to the Treasurer's report. The sum of $800 was provided by the Woods Hole Oceanographic Institution for library acquisitions.

During the year, 1209 (55 new) current journals were received. Of these, 329 (12 new) were Marine Biological Laboratory subscriptions, 510 (14 new) were exchanges, and 168 (3 new) were gifts; 51 (4 new) were Woods Hole Oceanographic Institution subscriptions, 137 (17 new) were exchanges, and 14 (5 new) were gifts. Due to the great irregularity in current receipts of German and Russian journals, most of these titles were omitted in this tabulation.

The Marine Biological Laboratory purchased 78 books, received 9 complimentary copies from authors, 43 gifts from the publishing firms, 152 books from the E. L. Mark Library, and 23 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 32 books. These made a total of 337 titles acquired.

There were 27 back sets completed: 18 by purchase (5 Woods Hole Oceanographic Institution), 6 by exchange (3 Woods Hole Oceanographic Institution), and 3 by gift (2 Woods Hole Oceanographic Institution); 55 were partially completed: 39 by purchase (9 Woods Hole Oceanographic Institution), 1 by exchange (Woods Hole Oceanographic Institution), and 15 by gift.

The reprint additions to the Library numbered 11,310. Of these, 1385 were of current issue and the others were of earlier dates. Of the 24,690 papers acquired through the collections of Drs. Ulrich Dahlgren, Alfred C. Redfield, Frank R. Lillie, and a gift from the Boston University School of Medicine, 4642 were found to be reprints not already appearing in the Library's collection.

A sum of $2989.85 from the Carnegie Corporation of New York Fund was spent for 6 books, 9 completed back sets, 16 partially completed back sets, and the binding of 337 volumes.

There were 86 microfilm orders filled during the year. Libraries and individuals have been encouraged to use this service in preference to requesting volumes on inter-library loan. In spite of this effort, 94 volumes were sent out on loan, and 51 were borrowed for the investigators.

Several valuable gifts were received in 1948. The most outstanding of these was the collection of Dr. Frank R. Lillie's reprints. Dr. Lillie's thought in requesting that this Library inherit his collection was one of the many kindnesses shown throughout the years of his association with this Laboratory. Grateful acknowledgment is also made to the Bermuda Biological Station for Research, Inc., for the gift of the E. L. Mark Library of books and reprints. Time has not permitted the complete assorting of this huge amount of material, but 152 books and 1600 reprints have been added to the shelves. Among these were several very valuable books by Louis Agassiz. The Library is also indebted to the American

Academy of Arts and Sciences for a four-volume set of Count Rumford's works.

The Library contained at the end of 1948, 57,548 bound volumes and 160,528 reprints.

Respectfully submitted,
DEBORAH LAWRENCE
Acting Librarian

VI. THE REPORT OF THE DIRECTOR

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY

Gentlemen:

I submit herewith a report of the sixty-first session of the Marine Biological Laboratory for the year 1948.

During the past year our physical plant has undergone many changes, chief of which are the renovation of Old Main; the purchase of the Elliott House; the purchase of the Dolphin; and the development of a laboratory for work with radioactive isotopes.

1. *Renovation of Old Main*

The generous gift of $150,000 made by the Rockefeller Foundation for the renovation of Old Main has enabled us to make necessary and long desired changes in that building. Preliminary plans for the work were prepared by the firm of Coolidge, Shepley, Bulfinch, and Abbott in consultation with the Building Committee consisting of Mr. Claff, Chairman, and Drs. Marsland, Parpart, and Packard. This Committee was later enlarged to include Messrs. MacNaught, Smith, Robert Kahler, and Pierce of the Laboratory Staff. Because of the high cost of construction not all of the changes desired by the Committee could be made. However, with the available funds, the building, which is structurally sound, can be put into excellent condition.

It was decided to construct a basement under the entire building during the Spring of 1949, and to finish the renovation of the upper floors after the close of the summer session. By this arrangement, the classes can be held as usual, without any interruption. The Spring operations are in charge of the Sawyer Construction Company which built the new wing of the Library. The basement contains ten research rooms, two general laboratories, all equipped with sea water tables, and other necessary facilities, three dark rooms with sea water tables, a cold room (to be completed later), and service rooms. Between the wings of the building a sunken court provides light for the rooms opening on to it, and space for two large fish pools. The old plumbing, for many years in deplorable condition, has been completely replaced in the basement, and will be greatly improved elsewhere after the close of the summer season. Additions to the northwest and southwest corners of the building give extra space in the basement and on the first and second floors. The total area of the basement plus these newly constructed rooms is about equal to one floor of the Crane Building. Thanks to the Rockefeller Foundation we shall have a laboratory which will adequately accommodate the classes and the instructors for many years.

Some of our workers have doubted the wisdom of spending $150,000 on an old building, while others are pleased that Old Main, where so much important biological research has been carried on during the past sixty years, will be used by new generations of students. While a completely new structure would be desirable, there seemed to be no prospect of obtaining funds for the construction and equipment. On the other hand, the old building is well worth preserving. Mr. Sawyer, under whose direction the renovation has been made, writes that "The existing walls, floors and roof construction in Old Main are in excellent condition, although certain parts of the wall and roof shingling must be renewed in the comparatively near future. In our opinion, you have done the economical thing in making use of as much of Old Main as is possible."

2. *The Elliott House*

As a result of the purchase of the Elliott House the Laboratory now owns all the property on both sides of Center Street. This house, which will accommodate about twenty people, has been improved by the addition of an upstairs bathroom, and other changes.

3. *The Dolphin*

The purchase of a new boat, in addition to the *Limulus,* was mentioned in the last Annual Report. The new craft, the *Dolphin,* was in service during the summer of 1948, taking the classes on field trips and making collections of living material. It accommodates seventy passengers, is seaworthy and fast, and is a welcome addition to the fleet. Its gasoline engine has now been replaced by a Diesel motor acquired from the War Assets Administration and installed by our machinist, Mr. Harlow.

4. *The Radiation Laboratory*

Under the direction of Dr. Failla, the Radiation Laboratory has been greatly expanded. The American Cancer Society last year contributed $4,300 for the purchase of measuring instruments and other apparatus, and for equipment needed to safeguard investigators using radio-active substances. A "hot laboratory" where these materials can be transferred is now furnished with special tables, cabinets, and a hood.

This new field of research has attracted many workers. During the summer of 1948 fifteen investigators were using radio-active isotopes, among them being four Fellows supported by funds granted by the Atomic Energy Commission.

To carry on this type of research successfully, investigators require special training. For this reason Dr. Failla arranged a course of lectures given by well known specialists. Dr. Paul Aebersold, Chief of the Isotopes Division at Oak Ridge, took an active part in the course, and was available for advice on technical problems.

5. *Winter Research*

During a large part of the year the Institute of Muscle Research, under the direction of Dr. Szent-Gyorgyi has carried on research here. The number of investigators has grown until now there are eight at work. The laboratory of Experi-

mental Cell Research, directed by Dr. Chambers, occupied rooms during the winter of 1947–48, but has now moved to New York City.

6. *Gifts*

The Rockefeller Foundation, in addition to its gift of $150,000 for the renovation of Old Main, has also granted for the general support of the Laboratory the sum of $100,000, available during the next five years at the rate of not more than $25,000 per year. This is most welcome. for we stand in need of funds to replace old equipment, especially in the Apparatus and Chemical Departments, and to purchase other apparatus required for research along lines not heretofore followed at this Laboratory. This gift, however, is not restricted to such purposes and can be used for other laboratory needs.

In his will, Dr. F. R. Lillie bequeathed to the Laboratory 500 shares of Crane Company stock, the book value of which is $17,250.

Other gifts have been received from

The Schwarzhaupt Foundation towards the purchase of a new boat	$1,000
Dr. W. D. Curtis, for a bandsaw in the Carpenter Shop	$225
Mr. Leo H. Spivack	$200
The M. B. L. Associates	$1,320
The American Cancer Society to purchase apparatus to be used in the Laboratory of Experimental Cell Research and for	$5,000
equipment and technical help in the Radiation Laboratory	$4,300

7. *Changes in Personnel*

Dr. E. P. Little, manager of the Apparatus Department since 1942, and of the Chemical Department during the war years, has resigned in order to carry on research at the Computation Laboratory of Harvard University.

During his incumbency he greatly enlarged the Apparatus Department and increased its efficiency. We are glad to retain him as a Consultant. The new manager is Mr. Robert Mills who has been associated with Dr. Little for some time. Mr. Gail Cavanaugh, head of the Science Department at the Falmouth High School, has been appointed manager of the Chemical Department.

We shall miss "Colonel" Wamsley who died in the spring of 1949. Coming here in 1892 as a student from Brown University he became a member of the Corporation. He was connected with the Charleston, S. C. Museum for many years, and served as a superintendent of one of the city schools. During the summers he was a special collector in the Supply Department.

8. *Election of Trustees*

At the meeting of the Corporation, August 10, 1948, the following trustees were elected.

Class of 1952

E. S. G. Barron
D. W. Bronk
G. Failla
C. O'D. Iselin
R. T. Kempton
C. W. Metz
Wm. Randolph Taylor
George Wald

Albert Tyler was elected to fill the vacancy caused by the death of S. C. Brooks of the Class of 1949.

H. H. Plough was elected in place of G. H. A. Clowes of the Class of 1951, who was made Trustee Emeritus.

9. *There are appended as parts of this report:*

1. The Staff
2. Investigators and Students
3. The Lalor Fellows
4. The Atomic Energy Commission Fellows
5. Tabular View of Attendance
6. Subscribing and Cooperating Institutions
7. Evening Lectures
8. Shorter Scientific Papers Presented at the Seminar
9. Members of the Corporation

Respectfully submitted,
CHARLES PACKARD,
Director

1. THE STAFF, 1948

CHARLES PACKARD, Director, Marine Biological Laboratory, Woods Hole, Massachusetts.

SENIOR STAFF OF INVESTIGATION

E. G. CONKLIN, Professor of Zoology, Emeritus, Princeton University.
RALPH S. LILLIE, Professor of General Physiology, Emeritus, The University of Chicago.
A. P. MATHEWS, Professor of Biochemistry, Emeritus, University of Cincinnati.
G. H. PARKER, Professor of Zoology, Emeritus, Harvard University.

ZOOLOGY

I. CONSULTANTS

LIBBIE H. HYMAN, American Museum of Natural History.
A. C. REDFIELD, Woods Hole Oceanographic Institution.

II. INSTRUCTORS

F. A. BROWN, Professor of Zoology, Northwestern University, in charge of course.
W. D. BURBANCK, Professor of Biology, Drury College.
C. G. GOODCHILD, Professor of Biology, S.W. Missouri State College.
L. H. KLEINHOLZ, Associate Professor, Reed College.
JOHN H. LOCHHEAD, Assistant Professor of Zoology, University of Vermont.
MADELENE E. PIERCE, Associate Professor of Zoology, Vassar College.
W. M. REID, Professor of Biology, Monmouth College.
T. H. WATERMAN, Assistant Professor in Biology, Yale University.

III. LABORATORY ASSISTANTS

R. S. HOWARD, University of Miami.
MARIE WILSON, Northwestern University.

EMBRYOLOGY

I. Consultants

H. B. Goodrich, Professor of Biology, Wesleyan University.
Albert Tyler, Associate Professor of Embryology, California Institute of Technology.

II. Instructors

Donald P. Costello, Prfoessor of Zoology, University of North Carolina, in charge of course.
Arthur L. Colwin, Assistant Professor of Zoology, Queens College.
Howard L. Hamilton, Associate Professor of Zoology, Iowa State College.
Charles B. Metz, Assistant Professor of Zoology, Yale University.

III. Research Assistant

Marjorie Hopkins Fox, University of California.

IV. Laboratory Assistant

Helen A. Padykula, Mount Holyoke College.

PHYSIOLOGY

I. Consultants

Eric G. Ball, Professor of Biochemistry, Harvard University Medical School.
Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania.
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine.
Arthur K. Parpart, Professor of Biology, Princeton University.

II. Instructors

E. S. Guzman Barron, Associate Professor of Biochemistry, The University of Chicago, in charge of course.
M. J. Kopac, Associate Professor of Biology, New York University.
John F. Muntz, Assistant Professor of Biochemistry, Western Reserve University Medical School.
Robert F. Pitts, Professor of Physiology, Syracuse University, College of Medicine.
H. Burr Steinbach, Professor of Zoology, University of Minnesota.
George Wald, Professor of Biology, Harvard University.
Dorothy Wrinch, Lecturer, Smith College.

BOTANY

I. Consultants

P. R. Burkholder, Eaton Professor of Botany, Yale University.
W. R. Taylor, University of Michigan.

II. Instructors

Maxwell S. Doty, Assistant Professor of Botany, Northwestern University. In Charge of Course.
H. C. Bold, Vanderbilt University.
R. D. Northcraft, Rutgers University.

III. Research Assistants

Justine Garnic, Carnegie Institute of Technology.
Leonard E. Spiegel, Drew University.

IV. Laboratory Assistant

Remi J. Cadoret, Harvard University.

V. Lecturers

J. B. Lackey, Philadelphia, Pa.
R. D. Wood, Rhode Island State College.

VI. Field Consultant and Collector

Edwin T. Moul, University of Pennsylvania.

EXPERIMENTAL RADIOLOGY

G. Failla, College of Physicians and Surgeons, Columbia University.
L. Robinson Hyde, Phillips Exeter Academy, Exeter, N. H.

LIBRARY

Deborah Lawrence, Acting Librarian
Margaret P. McInnis — Mary A. Rohan
Jean Goodfellow

APPARATUS DEPARTMENT

E. P. Little, Phillips Exeter Academy, Exeter, N. H., Manager
J. D. Graham — Dorothy LeFevre
Robert Mills

CHEMICAL DEPARTMENT

E. P. Little, Phillips Exeter Academy, Exeter, N. H., Manager

SUPPLY DEPARTMENT

James McInnis, Manager
John S. Rankin, Naturalist
Ruth Crowell — Marcia McLaughlin
M. B. Gray — W. E. Kahler — R. E. Tonks
A. M. Hilton — W. S. Landers — F. N. Whitman
G. Lehy

GENERAL OFFICE

F. M. MacNaught, Business Manager
Homer P. Smith, Assistant Business Manager
Polly L. Crowell — Mrs. Lila S. Myers

GENERAL MAINTENANCE

R. W. Kahler, Manager

Robert Adams — A. Neal
R. Gunning — G. T. Nichelson, Jr.
J. H. Head — A. J. Pierce
G. A. Kahler — T. E. Tawell
Seaver R. Harlow

THE GEORGE M. GRAY MUSEUM

2. Investigators and Students

Independent Investigators, 1948

Abelson, Philip H., Staff Member, Carnegie Institute of Washington.
Abrams, Richard, Assistant Professor, University of Chicago.
Aebersold, Paul C., Chief, Isotopes Branch, Atomic Energy Commission.
Agersborg, H. P. K., Professor of Histology, Des Moines Still College.
Allen, M. Jean, Instructor in Biology, Mather College.
Amberson, William R., Professor of Physiology, University of Maryland Medical School.
Ardao, Maria Isabel, University of Montevideo.
Armaghan, Veronica, New York City.
Armstrong, Philip B., Professor of Anatomy, College of Medicine, Syracuse University.
Augustinsson, Klas-Bertil, University of Stockholm.
Baker, Gladys E., Associate Professor of Plant Science, Vassar College.
Baldwin, Ernest H. F., University Lecturer in Biochemistry, Cambridge, England.
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School.
Barron, E. S. Guzman, Associate Professor of Biochemistry, University of Chicago.
Berger, Charles A., Director, Biological Laboratory, Fordham University.
Bliss, Alfred F., Assistant Professor of Physiology, Tufts Medical School.
Blum, Harold F., Physiologist, National Cancer Institute.
Bonner, John T., Assistant Professor of Biology, Princeton University.
Bowers, John Z., Assistant and Director and Chief, U. S. Atomic Energy Commission.
Boyer, Donald D., Instructor in Biology, Union College.
Bridgman, Anna J., Professor of Biology, Limestone College.
Brooks, Matilda, Professor of Zoology, University of California.
Brown, Frank A., Jr., Professor of Zoology, Northwestern University.
Burbanck, William D., Professor of Biology, Drury College.
Butler, Elmer G., Professor of Zoology, Princeton University.
Cameron, Gladys, Research Associate, New York City.
Cannan, R. Keith, Professor of Chemistry, New York University College of Medicine.
Chambers, Robert, Chief, Laboratory of Experimental Cell Research.
Chance, Britton, Assistant Professor of Biophysics, Johnson Research Foundation.
Chase, Aurin M., Associate Professor of Biology, Princeton University.
Cheney, Ralph H., Associate Professor of Biology, Brooklyn College.
Chrystall, Frieda L., Teacher of Biology, Julia Richman High School.
Chu, Hao-jan, Graduate Assistant in Botany, Northwestern University.
Claff, C. Lloyd, Research Fellow in Surgery, Harvard Medical School.
Clark, Arnold M., Instructor in Biology, University of Delaware.
Clark, Eliot R., Professor of Anatomy, University of Pennsylvania School of Medicine.
Clark, Leonard B., Professor of Biology, Union College.
Claude, Albert, Associate Member, The Rockefeller Institute.
Clement, A. C., Professor of Biology, College of Charleston.
Clowes, G. H. A., Research Director Emeritus, Lilly Research Laboratories.
Cohen, Isadore, Assistant Professor, American International College.
Cole, Kenneth S., Professor of Biophysics, University of Chicago.
Colwin, Arthur L., Assistant Professor of Biology, Queens College.
Conklin, Edwin G., Professor of Biology Emeritus, Princeton University.
Cooper, Kenneth W., Associate Professor of Biology, Princeton University.
Copeland, E. Eugene, Assistant Professor of Biology, Brown University.
Cornman, Ivor, Research Fellow, Sloan-Kettering Institute.
Costello, Donald P., Professor of Zoology, University of North Carolina.
Coyle, Elizabeth E., Assistant Professor of Biology, College of Wooster.
Crowell, Sears, Associate Professor of Zoology, Miami University.
Dent, J. N., Assistant Professor, University of Pittsburgh.
Diller, Irene C., Research Cytologist, Institute for Cancer Research, Lankinau Hospital.

DILLER, WILLIAM F., Assistant Professor of Zoology, University of Pennsylvania.
DOTY, MAXWELL S., Assistant Professor of Botany, Northwestern University.
DURYEE, WILLIAM R., Research Associate, Carnegie Institution of Washington.
EDGERLEY, ROBERT H., Research Scientist, Columbia University.
EICHEL, BERTRAM, Research Associate, New York University College of Dentistry.
EVANS, TITUS C., Research Professor of Radiology, State University of Iowa.
FAHEY, ELIZABETH M., Teacher, Taunton High School.
FAILLA, G., Professor of Radiology, Columbia University.
FLOOD, VERONICA M., Junior Scientist, Argonne National Laboratories.
FRENKEL, ALBERT W., Assistant Professor of Botany, University of Minnesota.
FROEHLICH, ALFRED, Associate, The May Institute for Medical Research.
GAFFRON, HANS, Associate Professor of Biochemistry, University of Chicago.
GERARD, POL, Professor of Pathology, The Belgian American Educational Foundation.
GLASER, O. C., Professor of Biology, Amherst College.
GOLDSTEIN, AVRAM, Instructor in Pharmacology, Harvard University Medical School.
GOODCHILD, C. G., Professor of Biology. Missouri State College.
GOULD, HARLEY N., Professor of Biology. Tulane University, Newcomb College.
GRAND, C. G., Research Associate, New York University.
GRAY, IRVING M., Professor of Zoology, Duke University.
GREENBERG, G. ROBERT, Senior Instructor in Biochemistry, Western Reserve University.
GREGG, JOHN R., Assistant Professor of Zoology, Columbia University.
GROSCH, DANIEL S., Assistant Professor of Zoology, North Carolina State College.
GRUNDFEST, HARRY, Assistant Professor of Neurology, Columbia University.
HAMILTON, HOWARD L., Associate Professor of Zoology, Iowa State College.
HARVEY, E. NEWTON, Professor of Physiology, Princeton University.
HARVEY, ETHEL BROWNE, Independent Investigator, Princeton University.
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HEIDENTHAL, GERTRUDE, Assistant Professor of Biology, Russell Sage College.
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KEMPTON, RUDOLF T., Professor of Zoology, Vassar College.
KISCH, BRUNO, Professor, Yeshiva University.
KITCHIN, IRWIN C., Associate Professor of Biology, University of Georgia.
KLEINHOLZ, LEWIS H., Associate Professor of Biology, Reed College.
KLOTZ, IRVING M., Associate Professor of Chemistry, Northwestern University.
KOPAC, M. J., Associate Professor of Biology, New York University.
KRAHL, MAURICE E., Assistant Professor of Pharmacology, Washington University.
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LEFEVRE, PAUL G., Assistant Professor of Physiology, University of Vermont.
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O'Brien, John A., Assistant Professor of Biology, Catholic University of America.
Olson, Magnus, Assistant Professor of Zoology, University of Minnesota.
Oster, Robert H., Professor of Physiology, University of Maryland.
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Scott, Sister Florence Marie, Professor of Biology, Seton Hill College.
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BULLOCH, JANE ANN, Student, University of Oklahoma.
COHEN, ARTHUR I., Student, University of Minnesota.
COOPERSTEIN, SHERWIN J., Student, New York University College of Dentistry.
CORET, IRVING A., Research Fellow, University of Pennsylvania.
EICHEL, HERBERT J., Graduate Student, New York University.
ESSNER, EDWARD S., Graduate Student, University of Pennsylvania.
FITCH, NAOMI, Assistant in Zoology, Columbia University.
GAGNON, ANDRE, Research Assistant, University of Pennsylvania.
GASVODA, BETTY M., Junior Scientist, Argonne National Laboratories.
GOODKIND, M. JAY, Student, Princeton University.
GOREAU, THOMAS F., Medical Student, University of Pennsylvania.
GREEN, JAMES W., Research Associate, Princeton University.
GREGG, JAMES H., Graduate Student, Princeton University.
HARDING, CLIFFORD V., JR., Graduate Student, University of Pennsylvania.
HAY, ELIZABETH D., Student, Smith College.
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HODGSON, EDWARD S., JR., Junior Instructor, Johns Hopkins University.
HOFFMAN, JOSEPH F., Student, University of Oklahoma.
HONEGGER, C. M., Instructor, Temple University.
HOPKINS, AMOS L., JR., Graduate Student, University of Pennsylvania.
JACOBSON, JULIUS H., Graduate Student, University of Pennsylvania.
JONES, GWEN MAXINE, Teaching Assistant, Northwestern University.
KELLY, JOHN W., Graduate Student, University of Pennsylvania.
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MARTIN, BARBARA ADELE, Assistant in Zoology, Barnard College.
MOSKOVIC, SAMUEL, Teaching Fellow, Graduate School, New York University.
NARDONE, ROLAND M., Laboratory Assistant, Fordham University.
NELSON, THOMAS CLIFFORD, Lecturer in Biophysics, Columbia University.
PADYKULA, HELEN A., Instructor, Wellesley College.
RAY, DAVID T., Graduate Student, University of Pennsylvania.
RIESER, PETER, Assistant Instructor, University of Pennsylvania.
ROSENBAUM, JOAN, Student, Columbia University.
ROSENBLUTH, RAJA, Graduate Student, Columbia University.
ROSSETTI, FIAMMETTA, Student, University of Chicago.

Rothberg, Harvey, Jr., Undergraduate, Princeton University.
Seaman, Gerald R., Graduate Assistant in Biology, Fordham University.
Susca, Louis A., Instructor, Fordham University.
Sze, L. C., Research Assistant, Columbia University.
Wilson, Walter L., Research Assistant, University of Pennsylvania.

Research Assistants, 1948

Baily, Norman A., Research Scientist, Columbia University.
Benson, Eleanore, Research Assistant, University of Missouri.
Berman, Jack H., Fellow in Anatomy, Western Reserve University.
Blumenthal, Gertrude, Research Associate, University of Missouri.
Clark, Grace, Laboratory Technician, Columbia University.
Cooper, Octavia, Research Assistant, Harvard University Medical School.
Crane, Robert K., Chemist, Eli Lilly and Company.
Curtis, Paul, Student, Bethany College.
Eggers, Annette, Acting Instructor, Stanford University.
Fass, Gerome S., Research Technician, Rockefeller Institute for Medical Research.
Foley, Mary T., Research Assistant, Yale University.
Fusco, Edna Marie, Research Assistant, Yale University.
Garnic, Justine, Graduate, Carnegie Institute of Technology.
Gordon, Marcia, Research Assistant, Harvard Medical School.
Henley, Catherine, Research Assistant, University of North Carolina.
Hickson, Anna Keltch, Research Chemist, Eli Lilly and Company.
Himmelfarb, Sylvia, Research Assistant, University of Maryland Medical School.
Howard, Robert S., Graduate Assistant, University of Miami.
Jacob, Miriam, Technician, Rockefeller Institute.
Keeney, Belle C., Stanford University.
Kimberly, Paul E., Associate Professor, Des Moines Still College.
Kirk, Marjorie J., Student, Harvard Medical School.
LeFevre, Marian E., Graduate Student, University of Vermont.
Lemm, Frances J., Research Assistant, Western Reserve University.
Lesse, Henry, Medical Student, Jefferson Medical College.
Litovchick, Mortimer, Research Technician, Rockefeller Institute.
Love, Lois H., Instructor, University of Pennsylvania.
Love, Warner E., Assistant Instructor, University of Pennsylvania.
Lowens, Mary D., Research Assistant, Harvard Medical School.
Metcalf, John S., Jr., Medical Student, University of Maryland School of Medicine.
Mitchell, Constance, Instructor, University of Delaware.
Nach, Lucille, Research Assistant, Western Reserve University.
Passano, Leonard M., III, Graduate Student, Yale University.
Podolsky, Betty, Research Assistant, University of Chicago.
Raskind, Josephine B., Bryn Mawr College.
Rawley, June, Student, University of Oklahoma.
Rich, Alexander, Research Assistant, Harvard Medical School.
Roth, Alexander, Research Assistant, University of Kansas.
Rothenberg, Mortimer A., Research Assistant, Columbia University.
Sandeen, Muriel I., Teaching Assistant, Northwestern University.
Seaman, Arlene R., Assistant, Cornell University.
Shedd, Donald H., Dartmouth College.
Slattery, Leo F., Research Assistant, University of Chicago.
Spiegel, Leonard E., Graduate Assistant in Botany, Northwestern University.
Van Hoesen, Drusilla, Research Associate, University of Pennsylvania.
Volkman, Alvin, Student, University of Buffalo.
Walters, C. Patricia, Chemist Assistant, Eli Lilly and Company.
Webb, H. Marguerite, Teaching Assistant, Northwestern University.
Wetstone, Howard J., Research Assistant, Smith College.
Wilson, Marie, Teaching Assistant, Northwestern University.
Winblad, James N., Research Assistant, University of Kansas Medical School.

Library Readers, 1948

ABELL, RICHARD G., Resident in Psychiatry, College of Physicians and Surgeons.
ADLER, FRANCIS H., Professor of Ophthalmology, University of Pennsylvania.
ADLERSBERG, DAVID, Adjunct Physician, Mt. Sinai Hospital.
ANDERSON, RUBERT S., Assistant Professor of Physiology, University of Maryland Medical School.
BARTLETT, JAMES H., Professor of Physics, University of Illinois.
BERMAN, EVELYN M., Teacher, Montreal Protestant.
BLAIR, JOHN H, Assistant Professor of Physiology, University of Massachusetts.
BOROFF, DANIEL A., Research Bacteriologist.
CURTIS, W. C., Dean and Professor of Zoology Emeritus, University of Missouri.
DISCHE, ZACHARIAS, Assistant Professor of Biochemistry, College of Physicians and Surgeons.
EHRLICH, MIRIAM, Graduate Student, Yale University.
FOLEY, JOSEPH B., Graduate Student, Yale University.
FREUND, JULES, Chief, Public Health Research Institute of the City of New York.
GATES, R. RUGGLES, Research Fellow in Biology, Harvard University.
GRANT, MADELEINE P., Member of Science Faculty, Sarah Lawrence College.
GUDERNATSCH, FREDERICK, Retired Visiting Professor, New York University.
GUREWICH, VLADIMIR, Assistant Visiting Physician, Bellevue Hospital.
HANKE, HARRIETT, Teaching Fellow in Biology, New York University.
HENSLEY, RUTH A., Graduate Assistant in Zoology, University of Missouri.
HESS, ECKHARD H., The University of Chicago.
HILL, RUTH F., Assistant Physicist, Sloan-Kettering Institute.
HUTCHINGS, LOIS M., Instructor, Drew University.
KAPLAN, ANN E., Graduate Assistant, Mt. Holyoke College.
KAUPE, WALTER, Research Assistant, Massachusetts Institute of Technology.
KAUZMANN, WALTER, Assistant Professor of Chemistry, Princeton University.
KEANE, JOHN FRANCIS, Fordham University.
KEEFFE, MARY M., Assistant Professor of Biology, College of St. Thomas.
KRASNOW, FRANCES, Head of Department of Research, The Guggenheim Dental Foundation.
LEIGHTON, JOSEPH, Massachusetts General Hospital.
LEIKIND, MORRIS C., Head of Biology and Medicine Unit, Library of Congress.
LISI, ALFRED G., Instructor, Department of Pharmacology, Jefferson Medical College.
LOEWI, OTTO, Research Professor, New York University College of Medicine.
McDONALD, SISTER ELIZABETH SETON, Professor of Biology, Mt. St. Joseph College.
MARTIN, ARTHUR W., Associate Professor of Physiology, University of Washington.
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SCHILLER, PAUL H., Research Associate, Yerkes Laboratories.
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SMITH, SYDNEY, Research Associate, University of Rochester.
STAUFFER, ROBERT C., Assistant Professor of History of Science, University of Wisconsin.
STERN, KURT G., Adjunct Professor of Biochemistry, Polytechnic Institute.
SULKIN, S. EDWARD, Professor and Chairman. Southwestern Medical College.
TAGNON, HENRY J., Associate Member, Sloan-Kettering Institute.
THERMAN, OLAF, Director of Laboratories, Pennsylvania Hospital.
TUTELMAN, HARRIET, Graduate Student, Johns Hopkins University.
WILLIER, B. H., Professor of Zoology, Johns Hopkins University.
YNTEMA, CHESTER L., Associate Professor of Anatomy, Syracuse University Medical School.

Students, 1948

BOTANY

ABBOTT, ROBINSON S., Student, Bucknell University.
BATTLEY, EDWIN H., Student, Harvard College.

BERNATOWICZ, ALBERT J., Student, Clark University.
COWEN, NAOMI, Student, Cornell University.
DIAMOND, RUDOLPH, Student, Syracuse University.
DOE, BARBARA, Instructor in Biology, Loretto Heights College.
EISENMAN, GEORGE, Student, Harvard University.
HANDLER, HOPE S., Student, Smith College.
HORWITZ, LEONARD, Student, College of the City of New York.
LAY, KO KO, Student, Washington University.
PRODELL, RITA C., Student, Drew University.
RUMELY, JOHN H., Student, Oberlin College.
SPRINGER, HELEN V., Student, Vassar College.
UMANZIO, DR. CARL B., Professor of Bacteria and Public Health, Kirksville College of Osteopathy.
WILLIAMS, LOUIS G., Associate Professor of Biology, Furman University.

EMBRYOLOGY

ANAGNOSTIS, IRENE P., Student, New York University.
CLARK, EUGENIE, Research Assistant, American Museum of Natural History.
CLARK, EDWARD C., Student, University of Massachusetts.
DANES, BETTY, Student, Mount Holyoke College.
DANIELS, EDWARD W., Research Assistant, University of Illinois.
EASTERLING, GEORGE R., Assistant Professor of Biology, Kent State University.
HAFFNER, RUDOLPH E., Student, Yale University.
HEALY, EUGENE A., Student, Columbia University.
HEATH, H. DUANE, Student, University of Chicago.
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JAFFEE, OSCAR C., Student, New York University.
JASKOSKI, BENEDICT J., Student, University of Minnesota.
JONES, EDWARD E., Student, University of North Carolina.
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RHODES, STANLEY A., Graduate Assistant, Duke University.
ROSSETTI, FIAMMETA, Student, University of Chicago.
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SCHREIMAN, EVELYN S., Assistant Instructor, Rutgers University.
TODD, DORIS J., Student, Smith College.
WASHINGTON, DOROTHY A., Student, George Washington University.
WATSON, RUBY J., Student, Wheaton College.

PHYSIOLOGY

BAUER, M. H., Graduate Student, Princeton University.
BERNSTEIN, MAURICE H., Graduate Assistant, Washington University.
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HOFFMAN, JOSEPH F., Student, University of Oklahoma.

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Junqueira, Luiz C. U., Associate Professor, University of São Paulo, Brazil.
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Tarr, Elizabeth H., Student, Stanford University.
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ZOOLOGY

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Bellinger, Peter F., Student, Yale University.
Berger, Ruth, Undergraduate Student, Goucher College.
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Boucot, Arthur J., Student, Harvard University.
Brown, Harley P., Assistant Professor, University of Oklahoma.
Burch, Charles, Graduate Assistant, Cornell University.
Coady, Martha B., Student, Simmons College.
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Deardorff, Alice A., Graduate Assistant, Wesleyan University.
Eckl, Betty A., Student, Mount Holyoke College.
Etz, Monica Zelda, Undergraduate Student. Goucher College.
Ewing, Mary J., Student, Pennsylvania College for Women.
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O'Malley, Benedict B., Instructor in Botany and Anatomy, Fordham University.
Orski, Barbara, Student, Hunter College.
Peloquin, Stanley J., Student, Marquette University.

Perry, Thomas O., Student, Harvard University.
Provasoli, Luigi, Research Associate, Haskins Laboratories.
Reichart, Ruth, Student, Radcliffe College.
Rogers, Kay T., Teaching Fellow, Harvard University.
Sachs, Barbara C., Student, Oberlin College.
Shomay, David, Student, Long Island University.
Slobodkin, Lawrence B., Graduate Student, Yale University.
Stevens, Arthur L., Research Associate, University of Notre Dame.
Thomas, Lyell J., Jr., Student, Oberlin College.
Urey, Gertrude E., Student, Swarthmore College.
Willcox, Barbara L., Student, Oberlin College.
Wilson, Carolyn E., Graduate Assistant, State University of Iowa.
Wunder, Charles C., Student, Washington and Jefferson College.

3. The Lalor Fellows

Senior Fellow: Ernest Baldwin, University of Cambridge, England.
Junior Fellows: Avram Goldstein, Harvard Medical School.
Joseph Lein, Syracuse University.
Harold Persky, Michael Reese Hospital, Chicago.
Warren Yudkin, Yale Medical School.
Reappointments: Irving Klotz, Northwestern University.
Arnold Lazarow, Western Reserve.
Benjamin Libet, University of Chicago.
Claude Villee, Harvard Medical School.

4. The Atomic Energy Commission Fellows

Richard Abrams, Institute of Radiobiology, University of Chicago.
D. E. Copeland, Brown University.
T. P. Ting, University of Chicago.
J. J. Wolken, University of Pittsburgh.
A. A. Woodward, Brown University.

5. Tabular View of Attendance

	1944	*1945*	*1946*	*1947*	*1948*
Investigators—Total	193	212	267	299	326
Independent	112	138	175	182	183
Under instruction	11	10	29	36	42
Library readers	50	38	38	36	50
Research assistants	20	26	25	45	51
Students—Total	75	96	122	131	123
Zoology	37	55	57	55	54
Embryology	23	23	30	33	29
Physiology	10	13	26	26	25
Botany	5	5	9	17	15
Total Attendance	276	308	389	430	449
Less persons registered as both students and investigators	1			2	6
	275				443
Institutions Represented—Total	106	124	141	148	158
By investigators	74	100	102	114	117
By students	41	49	56	56	68
Schools and Academies Represented					
By investigators	1	2	2	1	3
By students	2	2		1	
Foreign Institutions Represented					
By investigators	2	1	7	7	8
By students	3		5	3	4

6. Subscribing and Cooperating Institutions, 1948

Cooperating Institutions

Amherst College
Boston Dispensary
Brooklyn College
Brown University
Bryn Mawr College
Carnegie Inst. of Washington
Catholic University of America
College of Mt. St. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Cornell University
Duke University
Fordham University
Goucher College
Harvard University
Harvard University Medical School
Johns Hopkins University
Johns Hopkins University Medical School
Johnson Foundation
Eli Lilly Company
Madison Foundation
Massachusetts Inst. of Technology
Miami University
Mount Holyoke College
Mount Sinai Hospital
New York University College of Medicine
New York University School of Dentistry
New York University Washington Square College
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Rockefeller Foundation
Rockefeller Institute for Medical Research
Seton Hill College
Sloan-Kettering Institute
Smith College
State University of Iowa
Syracuse University Medical School
Temple University
Tufts College Medical School
Union College
University of Chicago
University of Illinois
University of Kansas
University of Maryland School of Medicine
University of Michigan
University of Minnesota
University of Missouri
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Vermont Medical School
University of Virginia
Vassar College
Washington University
Washington Univ. School of Dentistry
Wesleyan University
Western Reserve University
Woods Hole Oceanographic Institution
Yale University

Subscribing Institutions

Argonne National Laboratory
Belgium American Educational Fund
California Institute of Technology
College of Charleston
College of the City of New York
DesMoines Still College of Osteopathy
Drury College
Elmira College
Georgetown University School of Medicine
Hunter College
Institute for Cancer Research
Institute for Muscle Research
Institute of Pennsylvania Hospital
W. K. Kellogg Foundation
Marquette University
National Cancer Institute
National Institute of Health
North Carolina State College of Agriculture & Engineering
Public Health Research Institute of New York City
Radcliffe College
Southwestern Medical College
University of Delaware
University of Massachusetts
University of Oklahoma
University of Pittsburgh
Wheaton College

7. The Friday Evening Lectures, 1948

Friday, July 2
Dr. William R. Duryee .... "The Structure and Function of Egg Nuclei."
Friday, July 9
Dr. J. T. Bonner .... "Morphogenetic Movements in the Amoeboid Slime Molds."

Friday, July 16
Dr. Ernest Baldwin .... "Nemathelminthes and Antihelminthics."

Friday, July 23
Dr. Paul C. Aebersold .... "Recent Developments in the Use of Isotopes in Biology and Medicine."

Friday, July 30
Dr. A. H. Sturtevant .... "Closely Similar Genes at Identical Loci."

Friday, August 6
Dr. John O. Hutchens .... "Biological and Chemical Activities of Nitrogen Mustards."

Friday, August 13
Prof. E. W. Sinnott .... "The Problem of Size Determination in Plants."

Friday, August 20
Dr. Paul Weiss .... "Nerve Growth and the Problem of Synthesis of Protoplasm."

Friday, August 27
Prof. Karl F. Bonhoeffer .... "The Mechanism of Chemical Rhythmical Reaction."

Other Lectures

Wednesday, August 4
Dr. L. V. Foster .... "Advance in Optical Instrumentation."

Thursday, August 12
Dr. George Lower .... "Kodachromes of Marine Life at Woods Hole and Bermuda."

Wednesday, August 18
Dr. P. S. Galtsoff .... "Biological Explorations in the Gulf of Panama."

8. Seminars, 1948

July 6
Arnold Lazarow .... "Sulfhydral Metabolism of the Beta Cell: Its Relation to the Development of Diabetes."
Shlomo Hestrin .... "Action Pattern of Crystalline Muscle Phosphorylase."
Rudolph Keller .... "Vital Staining in Combined Ultra Violet and Daylight."

July 13
Milton Levy and Evelyn Slobodiansky "The Arrangement of Amino Acids in Silk—an Application of Isotopic Derivative Analysis."
Anita Zorzoli .... "The Histochemical Localization of Alkaline Phosphatase in Mouse Bones of Different Ages."
B. Eichel, S. Cooperstein and W. W. Wainio .... "A Partial Separation of the Cytochromes."

July 20
K. B. Augustinsson .... "On the Specificity of Cholinesterase."
David Nachmansohn .... "Effect of Anticholinesterases in conduction."
M. A. Rothenberg .... "Rate of Electrolyte Penetration into the Nerve Interior."

July 27

F. W. Whiting and Bertina M. Blauch .... "Reproductive Economy and the Genetic Block to Free Oviposition in the Chalcidoid Wasp, Melittobia."

Luiz C. V. Junqueira .... "Biochemical and Histochemical Observation on the Sexual Dimorphism of Mice Submaxillary Glands."

Alfred E. Bliss .... "Extraction of Purified Squid 'Visual Purple.' "

James A. Miller .... "PH Estimations in Reconstituting Tubularia Stems."

August 3

Ivor Cornman .... "Inhibition of Sea Urchin Egg Cleavage by a Series of Substituted Carbamates."

Alfred Marshak .... "A Nuclear Precursor to Ribo- and Desoxyribonucleic Acids."

A. M. Shanes .... "The Effects of Excitatory and Blocking Mechanism Supporting the Resting Potential of Nerve."

August 10

James W. Green .... "The Relative Rate of Penetration of the Lower Fatty Acids into Beef Red Cells."

F. R. Hunter .... "Osmotic Hemolysis in Hypertonic Solutions."

Harold Persky .... "Hippuric Acid Excretion in Anxiety States."

Dorothy Wrinch .... "Biological Specificity."

August 17

R. K. Crane, E. G. Ball, and A. K. Solomon .... "Incorporation of Carbon Dioxide into Organic Linkage by Retina."

Claude A. Villee, M. Lowens, M. Gordon, E. Leonard, and A. Rich .... "The Synthesis of Nucleoproteins in Developing Arbacia Studied with the Aid of P32."

Avram Goldstein .... "Mechanisms of Interaction of Inhibitors with Plasma Cholinesterase."

E. DeRobertis .... "Ultrastructure of the Nerve Axon."

9. Members of the Corporation, 1948

1. Life Members

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Cole, Dr. Leon J., College of Agriculture, Madison, Wisconsin.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
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Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
King, Mr. Chas. A.
Kingsbury, Prof. B. F., Cornell University, Ithaca, New York.

Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
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Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pa.
Morgan, Mrs. T. H., Pasadena, California.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Scott, Dr. Ernest L., Columbia University, New York City, New York.
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Strong, Dr. O. S., Columbia University, New York City, New York.
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2. Regular Members

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.
Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.
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Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota.
Allee, Dr. W. C., The University of Chicago, Chicago, Illinois.
Amberson, Dr. William R., Department of Physiology, University of Maryland, School of Medicine, Baltimore, Md.
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Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
Angerer, Prof. C. A., Department of Physiology, Ohio State College, Columbus, Ohio.
Armstrong, Dr. Philip B., College of Medicine, Syracuse University, Syracuse, New York.
Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.
Baitsell, Dr. George A., Yale University, New Haven, Connecticut.
Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.
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Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.
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Barth, Dr. L. G., Department of Zoology, Columbia University, New York City, New York.
Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois.

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HANCE, DR. ROBERT T., The Cincinnati Milling Machine Co., Cincinnati 9, Ohio.

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Hayashi, Dr. Teru, Columbia University, New York City, New York.
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Hayes, Dr. Frederick R., Zoological Laboratory, Dalhousie University, Halifax, Nova Scotia.
Haywood, Dr. Charlotte, Mount Holyoke College, South Hadley, Massachusetts.
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Hisaw, Dr. F. L., Harvard University, Cambridge, Massachusetts.
Hoadley, Dr. Leigh, Biological Laboratories, Harvard University, Cambridge, Massachusetts.
Höber, Dr. Rudolf, University of Pennsylvania, Philadelphia, Pennsylvania.
Hodge, Dr. Charles, IV, Temple University, Department of Zoology, Philadelphia, Pennsylvania.
Hogue, Dr. Mary J., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Hollaender, Dr. Alexander, P.O. Box W., Clinton Laboratories, Oak Ridge, Tennessee.
Hopkins, Dr. Dwight L., University of Illinois, Pier Branch—Navy Pier, Division of Biological Science, Chicago, Illinois.
Hopkins, Dr. Hoyt S., New York University, College of Dentistry, New York City, New York.
Hunter, Dr. Francis R., Department of Zoology, University of Oklahoma, Norman, Oklahoma.
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Jones, Dr. E. Ruffin, University of Florida, Gainesville, Florida.
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Kempton, Prof. Rudolf T., Vassar College, Poughkeepsie, New York.
Kidder, Dr. George W., Amherst College, Amherst, Massachusetts.
Kidder, Jerome F., Woods Hole, Massachusetts.
Kille, Dr. Frank R., Carleton College, Northfield, Minnesota.
Kindred, Dr. J. E., University of Virginia, Charlottesville, Virginia.
King, Dr. Helen D., Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania.
King, Dr. Robert L., State University of Iowa, Iowa City, Iowa.
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STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS. IV. EFFECT OF X-RAY IRRADIATION ON THE RESPIRATION OF SEA URCHIN SPERM

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It has been maintained by a number of investigators (see reviews by Scott, 1937; Fricke, 1934; Packard, 1931) that the respiration of single cells is strikingly resistant to the action of ionizing radiations, and this belief has been the basis for ignoring the role of enzyme inhibition when explaining the mechanism of action of ionizing radiations on living cells. In fact, Chesley (1934) reported that the respiration of sea urchin eggs, both fertilized and unfertilized, was unaffected by x-ray irradiation with as much as 43,000 r. Henshaw (1932, 1940) found delay in cleavage whether the eggs or the sperm were irradiated, but the smallest amount of irradiation used in these experiments was 4000 r. Evans et al. (1942) confirmed Henshaw's work on sea urchin sperm and reported that inhibition of fertilization by x-rays could be partially prevented by the addition of certain organic substances, as had previously been found by Dale (1940) when he discovered this protective effect against enzyme inhibition by x-rays. In previous reports it has been shown that the respiration of tissue slices from rats irradiated with doses of x-rays below 500 r was definitely inhibited (Barron, 1946) and that the respiration of grasshopper eggs was inhibited with doses of x-rays as low as 10 r (Tahmisian and Barron, 1947). It was considered important, in view of these last experiments, to reinvestigate the problem of the respiration of irradiated single cells. We present in this paper data on the effect of x-rays on the respiration of sea urchin (*Arbacia punctulata*) sperm.

Experimental

Sperm was obtained by cutting circularly the soft tissues of the sea urchin. By this process sperm was shed in small syracuse dishes. Sperm from several urchins was collected in graduated centrifuge tubes after filtration through gauze. Filtered sea water was added to fill up the centrifuge tube. Sperm was then separated by short centrifugation (10 minutes at 2000 r.p.m.), the supernatant fluid was discarded, and the remaining sperm was brought to the desired dilution starting with a stock dilution of 1:10 or 1:20. The stock suspension was thoroughly shaken and an aliquot was taken for dry weight. To obtain the dry weights, 0.5 cc. of this suspension was added to specially hardened pyrex tubes and was centrifuged in a Beams type air-driven high speed centrifuge with 85 lbs. air pressure for 10 minutes. The fluid was withdrawn and the tubes were dried overnight at 110°. The sperm dilution chosen for the irradiation experiments was 1:200. This sperm suspension (1.2 cc.) was pipetted into small glass vials of 15 mm. diameter and 20 mm. height.

These vials (20 for each set of experiments) were placed in an aluminum holder resting in a glass container full of cracked ice. X-ray irradiation was performed by Mr. Hyde of the Department of Radiology of the Marine Biological Laboratory. The x-ray machine operated at 182 Kv peak voltage and a current of 25 ma. through each tube. A filter of 0.2 mm. Cu was used. The measurement of respiration started 40 minutes after irradiation.

Effect of dilution on the respiration of sea urchin sperm

Gray found in 1928 that the respiration of sea urchin sperm increased on dilution, but no quantitative study of this phenomenon has yet been reported. Although Hayashi (1946) attempted to measure this dilution effect, his techniques of measurement of O_2 uptake and of dilution were faulty, and his paper gives no data but rough figures. It was, therefore, necessary to determine the optimum sperm dilution which would give the maximum Q_{O_2} values (c.mm. of O_2 uptake per mg. dry weight per hour), and steady rates of respiration for at least one hour. A large number of experiments were performed for this purpose with different sperm dilutions, from 1:10 to 1:1000. The O_2 uptake increased steadily up to a dilution of 1:200. It started to decline when the dilution was increased to 1:400. A dilution of 1:1000

TABLE I

The effect of dilution on the respiration of sea urchin sperm (Arbacia punctulata). *Values, Q_{O_2}, give c.mm. O_2 uptake per mg. dry weight per hour*

Dilution	Q_{O_2}
1:10	1.7
1:30	3.6
1:100	10.0
1:150	14.6
1:200	19.6
1:400	17.5
1:1,000	2.0
1:1,600	None

gave an O_2 uptake as small as that of sperm at a dilution of 1:10; furthermore, the respiration almost ceased at the end of one hour. When the sperm was diluted to 1:1600, there was no measurable respiration (Table I). This lack of respiration was not due to lack of sensitivity of the Warburg manometric technique, for when measurements were made with the very sensitive Cartesian diver technique of Linderstrom-Lang as modified by Claff[1] similar negative results were obtained. The increase in respiration of sperm with dilution is undoubtedly due to greater motility in the dilute solutions.

When experiments were performed on one sample of pooled sperm, the values agreed within 10 per cent. The experiments performed on successive days with different sperm suspensions and the same dilution did not give reproducible values. The average Q_{O_2} value of 29 separate experiments performed on separate days (each experiment in triplicate) with a sperm dilution of 1:200 was 19.6 ± 3.9, i.e., with 20 per cent variation. Sperm dilutions of 1:200 could be kept at 3° for six hours with little decrease in respiration (11 to 14 per cent).

[1] Personal communication.

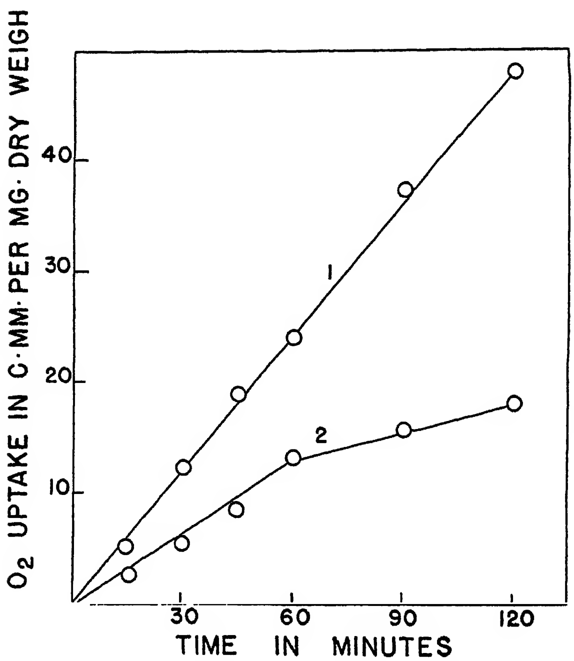

FIGURE 1. The effect of x-ray irradiation on the respiration of sea urchin sperm. Irradiation, 20,000 r. 1. Control; 2. Irradiated sperm.

It was not possible to obtain similar values in the O_2 uptake when the sperm was suspended in boiled sea water or in artificial sea water. The respiration of sperm in these cases was always much lower (20 to 40 per cent less).

Effect of x-rays on the respiration of sea urchin sperm

Once it was established that the optimum dilution for the measurement of sperm respiration was 1:200, all the experiments on the effect of x-ray irradiation were

TABLE II

Effect of x-rays on the respiration of sea urchin sperm. Dilution 1:200. Irradiation, 20,000 r. Figures give c.mm. O_2 uptake per mg. dry weight per hour

Exp. no.	Control	Irradiated	Inhibition (per cent)
1	14.8	6.0	59.4
2	16.2	3.8	76.6
3	12.0	5.0	58.4
4	23.9	8.9	62.8
5	18.3	5.3	71
6	13.1	2.8	79
7	29.6	17.6	54
8	18.6	10.6	43

performed with sperm suspensions so diluted. The sperm suspensions from 20 irradiated vials were collected in an Erlenmeyer flask from which 3 cc. were pipetted to each Warburg vessel. Thus, every experiment consisted of six control vessels and six vessels with irradiated sperm. Because of the reports in the literature on the resistance of respiration to x-ray irradiation, it was decided to start with 20,000 r. Such irradiation produced a marked inhibition of respiration, which increased in the second hour (Fig. 1). The degree of inhibition varied from sample to sample in all experiments. As an example of this variation the data of experiments of irradiation with 20,000 r are given in Table II. From this x-ray dose the amount of irradiation was diminished to 100 r with which an inhibition of 10 per cent was observed (Table III). The O_2 uptake inhibition on irradiation with 100 r did not increase in the second hour after irradiation (Fig. 2). In previous work

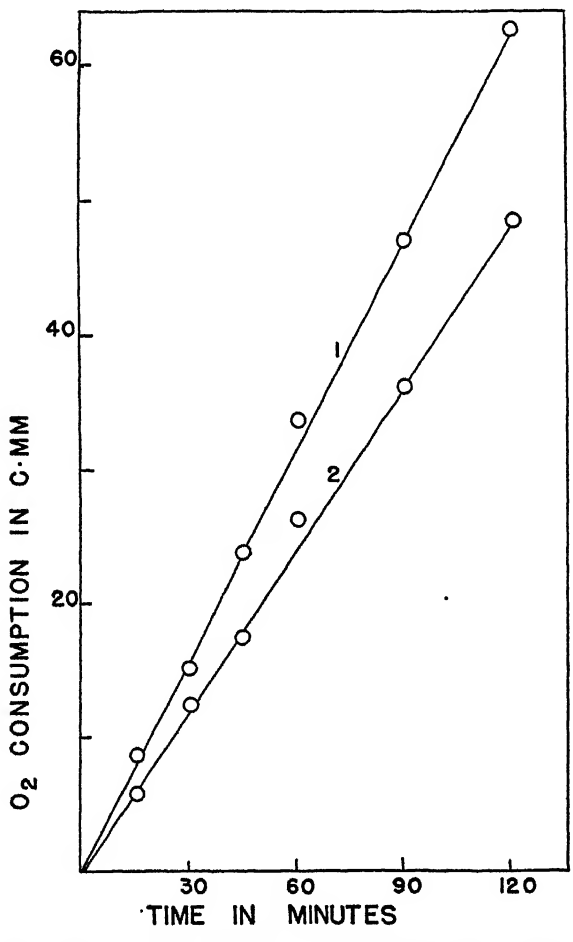

FIGURE 2. The effect of x-ray irradiation on the respiration of sea urchin sperm. Irradiation, 100 r. 1. Control; 2. Irradiated sperm.

TABLE III

The effect of x-rays on the respiration of sea urchin sperm. Sperm dilution 1:200. Q_{O_2} values, c.mm. O_2 uptake per mg. dry weight per hour

X-ray dose (r)	Q_{O_2} values		Inhibition (per cent)
	Control (c mm.)	X-ray (c.mm.)	
20,000	22	7.5	66
10,000	19	13.3	30
1000	28	19.8	22
500	18	15.5	14
100	20	18	10

with grasshopper eggs irradiated with small doses of x-rays, there was inhibition of respiration when measurement of the O_2 uptake was made soon after irradiation, but a return to normal values five hours after irradiation (Tahmisian and Barron, 1947). Several attempts were made to see whether the respiration of sea urchin sperm inhibited by x-rays could also recover a few hours after irradiation. Soon after irradiation the sperm-containing vials were kept at 3° for five hours and the respiration was then measured. The degree of inhibition was the same as that obtained when measurements were made soon after irradiation.

The inhibition of respiration produced by x-ray doses between 1000 r and 100 r could not be attributed to H_2O_2 formation (even if there were H_2O_2 formation on irradiation of sea water) because small amounts of H_2O_2 increased respiration. Furthermore, a portion of this H_2O_2 would be destroyed by sperm catalase (Fig. 3). The catalase content of sea urchin sperm was such that 1 mg. dry weight would produce 33 c.mm. O_2 per hour at 25°, a figure which is eleven times less than the catalase content of mouse testicle. For these experiments sperm was washed three times in sea water, it was suspended in 10 volumes of water, and homogenized. Sperm thus treated gave no O_2 uptake.

TABLE IV

Effect of x-rays on the oxidation of succinate and acetate by sea urchin sperm. Substrate concentration, 0.01 M

X-ray dose (r)	Substrate	Q_{O_2} values		Inhibition (per cent)
		Control (c.mm.)	Irradiation (c.mm.)	
100	None	22.2	19.3	13
100	Succinate	30.0	22.8	24
100	None	20.5	18.0	12
100	Acetate	25.0	18.3	27
1000	None	20.5	15.0	26.9
1000	Succinate	27.2	20.0	38

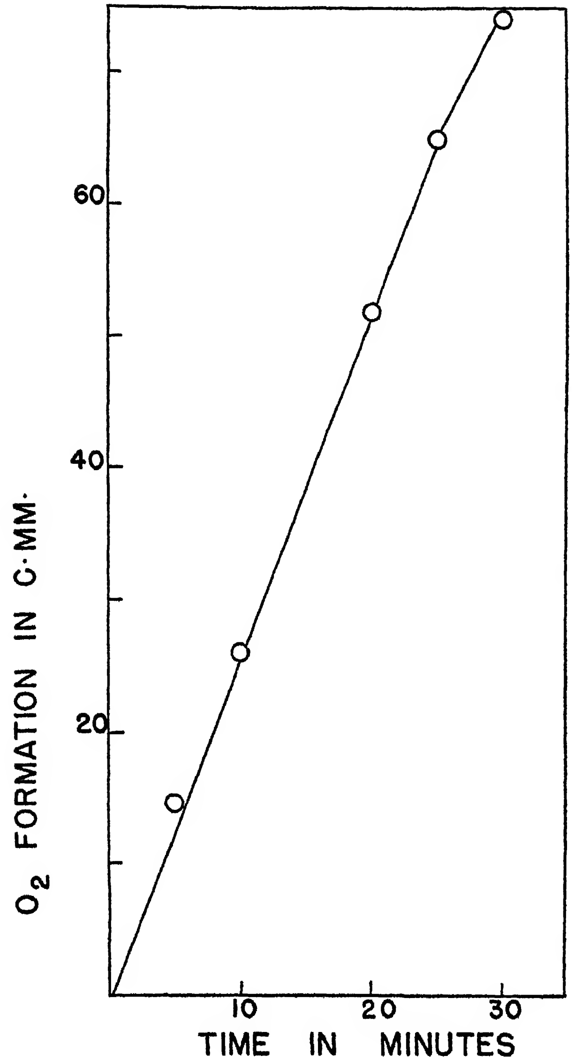

FIGURE 3. Catalase in sea urchin sperm. Buffer, phosphate, 0.01 M, pH 6.8; H_2O_2 0.01 M. Temperature 25°; Sperm, 0.1 cc. of 1:10 suspension in H_2O, 1.8 mg. dry weight.

A number of intermediary metabolites are utilized by sea urchin sperm with an increase in the O_2 uptake. Of these, the utilization of succinate and of acetate (as measured by the increase of O_2 uptake) was tested after irradiation with 100 r. In both cases the inhibition of O_2 uptake was greater in the presence of substrates (Table IV), an indication that the enzymes for the oxidation of succinate and of acetate which belong to the group of sulfhydryl enzymes, are quite sensitive to the inhibiting effect of x-rays.

Summary

The respiration of sea urchin (*Arbacia punctulata*) sperm increased with dilution up to a dilution of 1:200, where maximum values were found. At this dilution the average Q_{O_2} value was 19.6 ± 3.9. When the dilution was increased to 1:1000 the respiration dropped sharply to 2.0. A dilution of 1:1600 gave no measurable respiration.

The respiration of dilute suspensions of sea urchin sperm (1:200) was inhibited by x-ray irradiation. A dose of 20,000 r produced an inhibition of 66 per cent which was further increased during the second hour; 10,000 r inhibited 30 per cent; 1000 r, 22 per cent; 500 r, 14 per cent; and 100 r, 10 per cent. When sperm was irradiated with 1000 r there was no recovery of respiration five hours after irradiation. Inhibition of respiration cannot be attributed to hypothetical H_2O_2 formation, for sperm suspensions contain catalase. The catalase value of sperm is 33 c.mm. O_2 formed by 1 mg. dry weight per hour, i.e., 3 micromoles H_2O_2 destroyed. On addition of succinate and of acetate to sperm irradiated by x-rays the O_2 uptake inhibition increased.

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STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS. V. THE EFFECT OF HYDROGEN PEROXIDE AND OF X-RAY IRRADIATED SEA WATER ON THE RESPIRATION OF SEA URCHIN SPERM AND EGGS

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It has been known for some time (Risse, 1929; Fricke, 1934) that when water is irradiated with x-rays in the presence of oxygen there is formation of H_2O_2. Since H_2O_2 is a powerful oxidizing agent and it easily oxidizes sulfhydryl groups, it was reasonable to assume that this substance, if formed on irradiation, would contribute to the biological effects of ionizing radiations. In fact, Barron and Dickman (1949) on studying enzyme inhibitions by ionizing radiations, and Barron and Flood [1] on studying the oxidation of 2,3-dithiopropane and of glutathione by x-rays, were able to distinguish the H_2O_2 contribution to this oxidation by the use of catalase. The French investigators Loiseleur, Latarjet, and Caillot (1941), and Loiseleur and Latarjet (1942) have postulated that the primary effect of irradiation in aqueous solutions is H_2O_2 formation, which would thus become of importance in the interpretation of the mechanism of ionizing radiations. The same view is held by Evans (1947) who found that the fertilizing power of sea urchin sperm is decreased when suspended in sea water irradiated with large doses of x-rays. Evans attributed this inhibition to the action of H_2O_2 seemingly formed on irradiation of sea water. In living cells the role of H_2O_2 becomes more complicated because the sulfhydryl groups which might be oxidized by this agent not only are present in the protein moiety of enzymes, but also exist as non-protein sulfhydryl groups.

We present in this paper experiments on the effect of H_2O_2 and of x-ray irradiated sea water on the respiration of sea urchin sperm. They do not support the belief that H_2O_2 is an important factor in x-ray toxicity on sea urchin sperm.

Experimental

Sea urchin sperm was obtained as described previously (Barron et al., 1949), and in all experiments a dilution of 1:200 was used. Freshly filtered sea water was irradiated at room temperature in large cellophane dishes and immediately after irradiation the sperm suspension was added, enough to make the desired dilution of 1:200.

The catalase added to irradiated sea water was prepared from beef liver according to Sumner and Dounce (1939). H_2O_2 was determined by the colorimetric method of Bonet-Maury (1944). An aliquot of the solution was taken (up to

[1] Unpublished experiments.

3 cc.) to which was added 0.5 cc. of 20 per cent H_2SO_4, 5 drops of the titanium sulfate reagent (10 g. $TiSO_4$ ground in mortar with 50 cc. H_2O and 20 g. H_2SO_4, D = 1.84, let stand 24 h., centrifuge, take the supernatant and add 20 g. H_2SO_4), and distilled water to 5 cc. The yellow color produced which is stable was read in a Beckman spectrophotometer at 4000 Å. With this method amounts of H_2O_2 from 0.5 micrograms to 25 micrograms could be determined. The respiration measurements were made at 25°.

Effect of H_2O_2 on the respiration of sea urchin sperm

Barron et al. (1948) have shown that sulfhydryl reagents when used in small concentrations increase the respiration of sea urchin sperm, whereas they inhibit it when the concentration is increased. To explain these opposite effects it was postulated that the cell contains two kinds of sulfhydryl groups: the non-protein sulf-

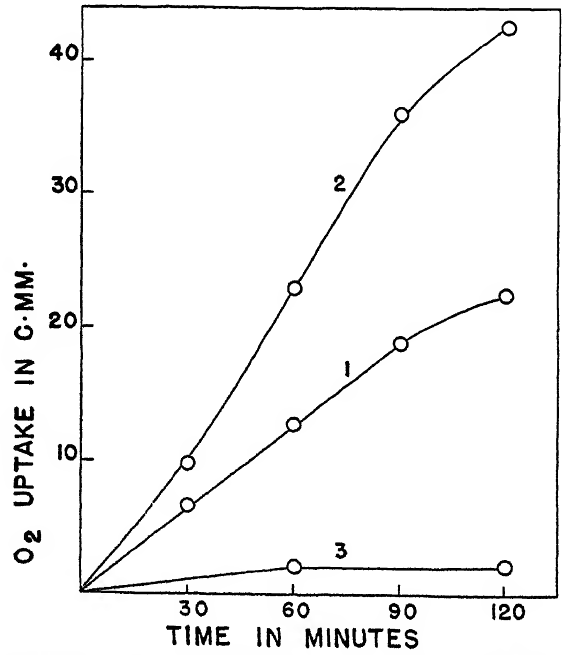

FIGURE 1. The effect of H_2O_2 on the respiration of sea urchin sperm. 1. Control; 2. H_2O_2, 1×10^{-5} M; 3. H_2O_2, 1×10^{-3} M.

hydryl groups (namely glutathione), which regulate the rate of respiration, and the sulfhydryl groups in respiratory enzymes. Destruction of the first would increase respiration, while destruction of the second would inhibit it. H_2O_2, an oxidizing agent of sulfhydryl groups, behaves in the same manner. At a concentration of 1×10^{-5} M it increased respiration, while 1×10^{-3} M inhibited it almost completely (Fig. 1). By altering the concentration of H_2O_2 between these two limits the effect on respiration is changed accordingly (Table I). In fertilized sea urchin eggs 1×10^{-4} M H_2O_2 increased the O_2 uptake 25 per cent (Fig. 2). If the effects of x-ray irradiation were mainly due to H_2O_2 formation, as postulated by

TABLE I

Effect of H_2O_2 on the respiration of sea urchin sperm. Sperm dilution, 1:200. Q_{O_2}, c.mm. O_2 uptake per mg. dry weight per hour

H_2O_2 Concentration (M)	Q_{O_2} values		Inhibition (−) or increase (+) (per cent)
	Control (c.mm.)	H_2O_2 (c.mm.)	
0.01	22.3	0	− complete
0.001	22.3	2.1	−90
0.0005	16.0	15.3	No effect
0.0001	20.2	33.7	+61.5
0.0001	22.0	42.0	+91

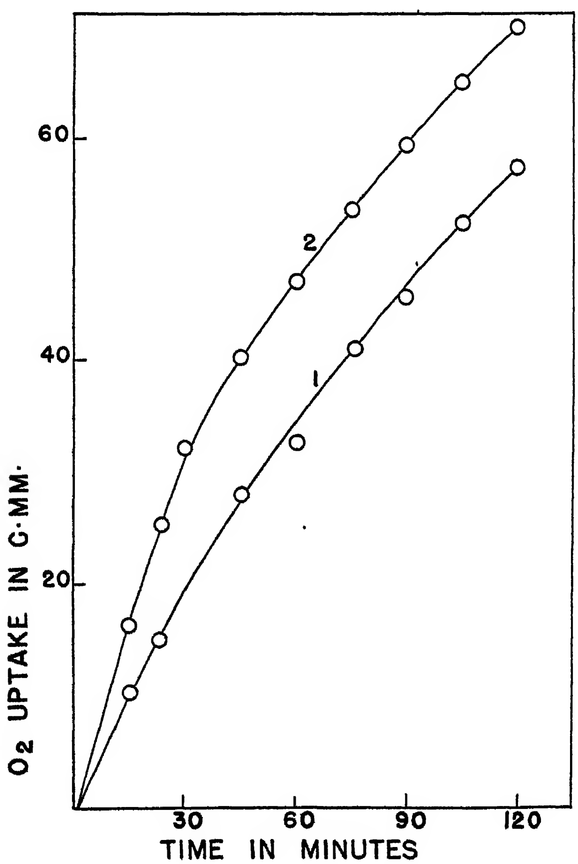

FIGURE 2. Effect of H_2O_2 on the respiration of fertilized sea urchin eggs. H_2O_2 concentration, 1×10^{-4} M; 1. Control; 2. H_2O_2.

TABLE II

Inhibition of sea urchin sperm respiration by x-ray irradiated sea water. X-ray dose, 100,000 r. Liver catalase (0.2 cc.) added immediately after irradiation; sperm suspension 5 minutes later

Experimental conditions	O_2 uptake		Inhibition (per cent)
	First hour (c.mm.)	Second hour (c.mm.)	
Control	29.7	53.5	
X-ray irradiated sea water	14.9	33.1	38
X-ray irradiation + catalase	16.2	36.6	34

Loiseleur et al. (1941, 1942) and by Evans (1947), x-ray irradiation at a dose of 1000 r would produce an increase in cell respiration.[2] Barron et al. (1949) have shown that on the contrary, respiration is inhibited. It must be concluded from these experiments that the effects of x-ray irradiation on the metabolism of sea urchin sperm cannot be attributed to H_2O_2 formation.

Effect of x-ray irradiated sea water on the respiration of sea urchin sperm

A number of investigators have reported that on irradiation of aqueous solutions, whether with x-rays or with ultra-violet light, there is formation of some unknown substance which will produce inhibition of growth of protozoa (Taylor et al., 1933),

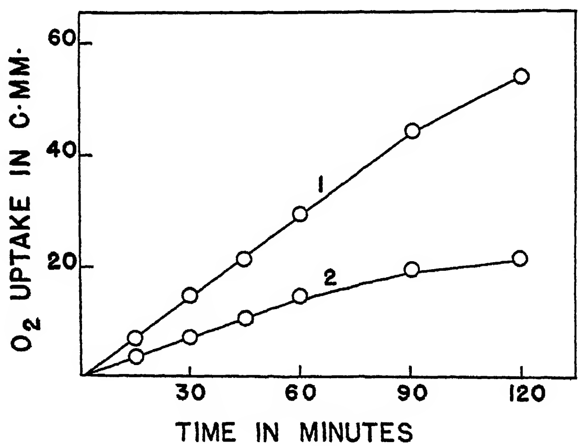

FIGURE 3. Inhibition of sea urchin sperm respiration by x-ray irradiated sea water. Irradiation, 200,000 r. 1. Sperm suspended in sea water; 2. Suspended in x-ray irradiated sea water.

inhibition of the fertilizing capacity of sea urchin sperm (Evans, 1947), inhibition of the growth of bacteria (Wyss et al., 1948), and increase in the mutation rate of *S. aureus* to penicillin resistance (Stone et al., 1947). When freshly filtered sea water was irradiated with 100,000 r, and a suspension of sperm was added to it soon

[2] From Bonet-Maury and Frilley's data (1944) it can be calculated that 1000 r would produce on irradiation of water about 1×10^{-5} M of H_2O_2.

after irradiation, there was an inhibition of respiration of 38 per cent. The inhibition was not affected by previous addition of catalase (Table II), an indication that the inhibition was not produced by H_2O_2. The inhibition increased when the dose of x-rays rose to 200,000 r (Fig. 3). Further evidence that this phenomenon was not produced by H_2O_2 was obtained by its detection with the titanous sulfate colorimetric method. While distilled water irradiated with 100,000 r gave 30 micrograms H_2O_2 per cc., filtered sea water irradiated with the same dose of x-rays gave no color at all. The lack of color formation was not due to the salt concentration of sea water, for on addition of H_2O_2 to sea water the color reaction appeared. We believe that the inhibition of respiration is due to the formation of stable organic peroxides formed on oxidation of the organic matter contained in sea water. It is quite possible that similar stable organic peroxides are formed on irradiation of biological fluids and that they contribute to the toxic effects of ionizing radiations. This problem is now under investigation.

Summary

Hydrogen peroxide at a concentration of 0.001 M produced almost complete inhibition of the respiration of sea urchin sperm suspended in sea water. At a concentration of 0.0005 M it had no effect. When the concentration was diminished to 0.0001 M it increased the respiration from 60 to 100 per cent.

When sperm was added to sea water irradiated with 200,000 r, there was a marked inhibition of respiration (about 60 per cent). Sea water irradiated with 50,000 r produced small inhibition (10 per cent). Addition of catalase previous to the addition of sperm had no effect at all on this inhibition. Furthermore, sea water irradiated with 200,000 r gave no positive test for H_2O_2. It is postulated that inhibition is due to the action of stable organic peroxides produced on irradiation of sea water.

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BACTERIA AND CELLULAR ACTIVITIES. IV. ACTION OF TOXINS ON RESPIRATION AND HEMOLYSIS OF DOGFISH ERYTHROCYTES AND ON RESPIRATION OF MARINE EGGS [1, 2]

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An attempt is being made in this laboratory to determine what effect bacterial toxins have on the functioning of cells. In the two preceding papers of this series (Hunter et al., 1949a, b) the action of seven toxins on the respiration and permeability of chicken erythrocytes was reported. In the present investigation the erythrocytes of the smooth dogfish, *Mustelus canis* and the unfertilized eggs of *Arbacia punctulata* and of *Asterias forbesei* were the cells studied.

Materials and Methods

The toxins were those previously used which were obtained locally and from the Lilly Laboratories and the Lederle Laboratories, to which the authors are indebted. Bacteriologically sterile techniques were used throughout except during the few minutes when hemolysis measurements were made. Tests for sterility were run at the end of each experiment as previously described (Hunter et al., 1949a). In addition, tests were also made for possible contamination by marine bacteria (see Waksman et al., 1933).

Oxygen consumption measurements were made using a Warburg apparatus at a temperature of 25° ± 0.1° C. Hemolysis times were measured using a photronic cell apparatus and a microammeter, since the more sensitive apparatus usually employed was not available. These measurements were made at room temperature which varied from day to day, but a water jacket surrounding the hemolysis chamber tended to minimize fluctuations during a series of readings. The time for hemolysis in 0.95 M ethylene glycol was measured in all cases.

Blood was procured under sterile conditions either by removal from the caudal vein with a hypodermic syringe, or by cutting the tail and allowing the blood to drain into a flask. Heparin was used as an anticoagulant in all experiments.

Immediately following the withdrawal of blood it was centrifuged at about 2000 r.p.m. for 10 minutes. The plasma and leucocytes were removed and the cells were carefully stirred. To 1 cc. of toxin was added 0.3 or 0.5 cc. of erythrocytes in each Warburg vessel which was immediately connected to its manometer and placed in the bath. Five to ten minutes were allowed for temperature equilibration, so that the initial readings were taken within 20 minutes or less of the time the toxins and cells were mixed. Controls were run using 1 cc. of sea water, 1 cc. of broth,

[1] This work was supported in part by grants from the Division of Grants and Research, U. S. Public Health Service and the Faculty Research Fund of the University of Oklahoma.

[2] An abstract of a portion of this work appeared in the *Biol. Bull.*, 95: 255, 1948.

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or 1 cc. of formalized toxins in place of the 1 cc. of toxins. In some experiments readings were made continuously, while in others the manometers were opened for about an hour following the first hour's readings. Cell counts and hematrocrit determinations were made as previously described.

Hemolysis measurements were made as follows: Equal volumes of blood and toxins were mixed and allowed to stand at room temperature. Controls were run substituting sea water, formalized toxins, formalized broth and formalized sea water for the toxins. From time to time 0.1 cc. aliquots were removed and added to 10 cc. of 0.95 M ethylene glycol in the hemolysis chamber. The course of hemolysis was followed by reading the microammeter at five-second intervals. The total exposure time to the toxins varied, but the maximum was 48 hours.

Formalized "controls" were used in both the respiratory and hemolysis experiments. These were the same stock suspensions previously described (Hunter et al., 1949a). As was true in the preceding work (Hunter et al., 1949b), these attenuated toxins were more satisfactory as "controls" for the permeability studies than for the respiratory studies. However, these attenuated toxins were more satisfactory as "controls" in the present respiration studies. Apparently the oxygen consumption of dogfish erythrocytes is not inhibited by formalin to such an extent as the oxygen consumption of chicken erythrocytes.

Unfertilized eggs of *Arbacia punctulata* and of *Asterias forbesei* were obtained under bacteriologically sterile conditions, using a technique similar to that described by Tyler et al. (1938). The dry weight of the eggs was obtained by centrifuging 1 cc. samples of the egg suspensions using an air turbine, removing the supernatant fluid and drying to constant weight.

In the majority of experiments in which eggs were used, 1 cc. of the toxin was placed in each Warburg vessel, 2 cc. of an egg suspension were added, the vessels were attached immediately to the manometers and five minutes were allowed for temperature equilibration. The first reading was taken within 20 minutes of the time the first vessel was prepared. Readings were taken at 10 minute intervals over a period of several hours.

RESULTS

Erythrocyte respiration

The results of a typical experiment in which dogfish erythrocytes were exposed to the toxins are summarized in Table I. Respiration was also measured in the presence of formalized toxins (26 days after mixing formalin and toxin). In the case of the three toxins which accelerate respiration (*M. aureus, Cl. tetani* and *C. diphtheriae*), cells in the presence of the corresponding formalized toxins consumed oxygen at essentially the same rate as sea water controls. The other formalized toxins inhibited the rate of respiration but none more markedly than formalized sea water.

Hemolysis times

The effects of the toxins on hemolysis of dogfish erythrocytes in 0.95 M ethylene glycol are shown in Table II. It can be seen that the toxins of *Cl. perfringens* are most effective in altering the surface of the cells, for a half hour exposure is sufficient to bring about a marked increase in the rate of hemolysis. A 9 hour ex-

TABLE I

A typical experiment showing the rates of respiration of dogfish erythrocytes in the presence of various toxins

Toxin	μL O_2 per cc. of cells per hour		Remarks	
	1st hour	3–5 hours	1st hour	3–5 hours
Control	80	70	———	———
M. aureus	140	108	Marked acceleration	Marked acceleration—slightly less than 1st hour
Cl. tetani	160	85	Marked acceleration	Slight acceleration
C. diphtheriae	120	75	Marked acceleration	Little or no effect
Cl. septicum	80	70	No effect	No effect
Cl. perfringens	80	0	No effect	Complete inhibition (Possibly associated with hemolysis of the cells)
B. cereus	65	60	Slight inhibition	Slight inhibition
Str. pyogenes	50	<20	Moderate inhibition	Marked inhibition

posure to the toxins of *Str. pyogenes* or *B. cereus* or a 23 hour exposure to the toxins of *Cl. tetani* produce a less marked increase in the rate of hemolysis. The formalized tetanal toxins had the same effect as the toxins themselves, while the formalized streptococcal toxins, and to a lesser extent, the formalized cereus toxins, had an intermediate effect. These data might indicate either that the formalin had not completely inactivated the toxins responsible for the change in the surface of the cells, or that the changes in the cells are brought about by something other than the toxins. Although additional experiments would be required to demonstrate conclusively the explanation for these observations, there is little reason for believing that substances other than the toxins would be influencing the cells in this manner. The composition of the broth in this case is unknown. However, none of the broths studied in this laboratory has had any observable influence on the osmotic behavior

TABLE II

Hemolysis times for dogfish erythrocytes in 0.95M ethylene glycol following exposure to various toxins

Time of exposure in hours	Organism producing toxin	Time in seconds for approximately 75 per cent hemolysis			
		Toxin	Control		
			Sea water	Formalized sea water	Formalized toxin
½	*Cl. perfringens*	11	31	31	29
9	*Str. pyogenes*	20	35	35	24
9	*B. cereus*	21	35	37	29
23	*Cl. tetani*	24	36	38	25
20	*Cl. septicum*	40	40	—	40
20	*C. diphtheriae*	30	35	25	30
20	*M. aureus*	35	35	25	30

of dogfish or other erythrocytes. In the case of tetanal toxin, the data obtained from the oxygen consumption studies suggest that the formalin does inactivate the toxin responsible for the change in respiration. As a tentative suggestion, therefore, one might assume that at least two tetanal toxins are present—one which accelerates respiration and is inactivated by formalin, and a second which alters the surface of the cell but which is not inactivated by formalin. It is hoped that future investigations will test the validity of this assumption.

The toxins of *Cl. septicum, C. diphtheriae,* and *M. aureus* have no effect on hemolysis following exposures of 20 hours.

Egg respiration

Figures 1 and 2 present the results of typical experiments using Arbacia and Asterias eggs respectively.

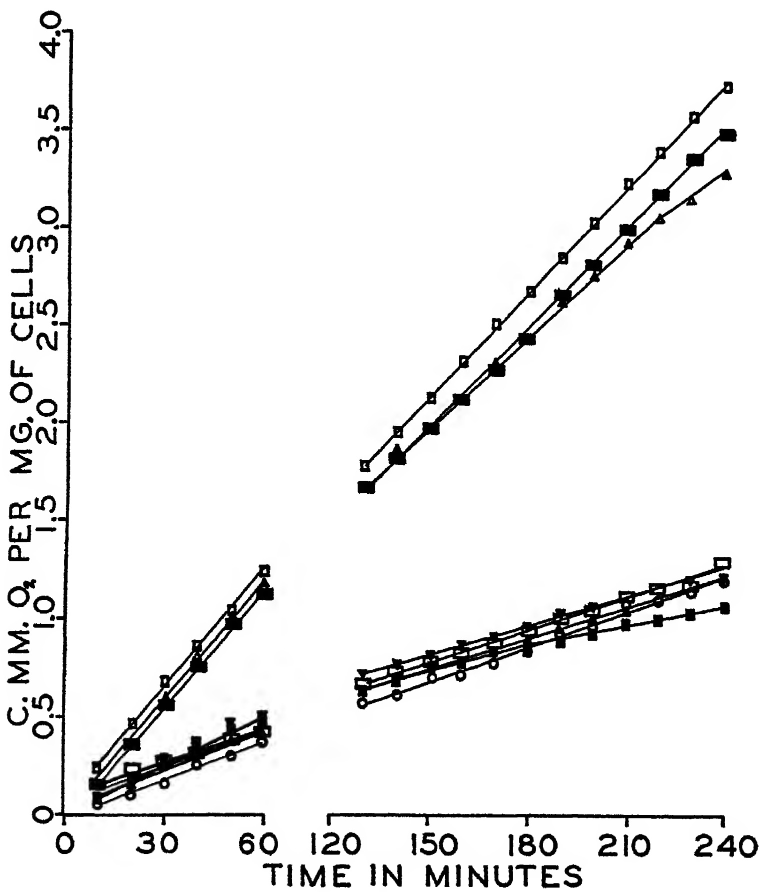

FIGURE 1. The effect of toxins on the oxygen consumption of unfertilized Arbacia eggs. Readings were taken for 60 minutes, then there was a 60 minute break and readings were again taken beginning at 120 minutes. □—*M. aureus;* ■—*Cl. tetani;* △—*C. diphtheriae;* ○—Sea water; ▽—*Cl. septicum;* ▲—*B. cereus;* ■—*Str. pyogenes;* □—*Cl. perfringens.*

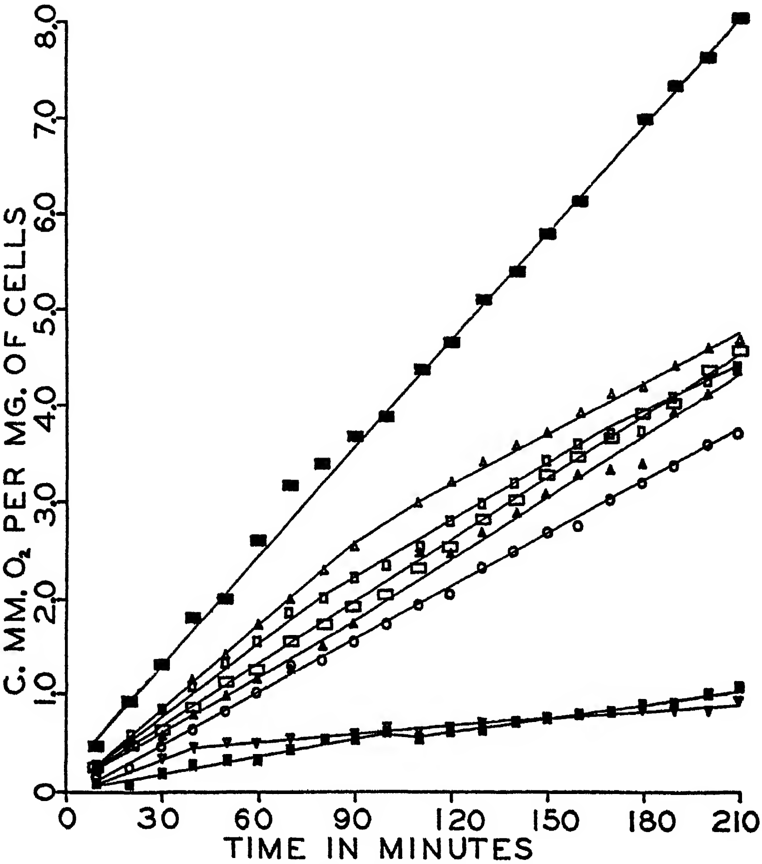

FIGURE 2. The effect of toxins on the oxygen consumption of unfertilized Asterias eggs. Readings were taken continuously for 210 minutes. ■—*Cl. tetani*; △—*C. diphtheriae*; ▭—*Cl. perfringens*; □—*M. aureus*; ▲—*B. cereus*; ○—Sea water; ■—*Str. pyogenes*; ▽—*Cl. septicum.*

These data are not conclusive since the heated toxins apparently had an effect similar to that of the toxins. However, it is interesting to note that the effects of these toxins on the eggs are in many respects the same as those on erythrocytes. In the case of Arbacia eggs, the toxins from *C. diphtheriae, Cl. tetani,* and *M. aureus* accelerate respiration. The significance of the lack of inhibition by the streptococcal toxins will be discussed in a future publication. In the case of Asterias eggs, the tetanal toxins clearly accelerate respiration; the diphtherial and staphylococcal toxins may accelerate slightly for the first hour or two; the perfringens toxins may accelerate slightly, while there is a suggestion of a delayed acceleration in the case of the toxins of *B. cereus.* The streptococcal toxins and those of *Cl. septicum* inhibit respiration.

Discussion

A comparison between these data and those previously reported (Hunter et al., 1949a, b) shows that in general the action of the toxins on the four types of cells

studied is essentially the same. Marked acceleration of respiration was obtained only with micrococcal, tetanal and diphtherial toxins. The fact that in the presence of micrococcal toxins the respiration of chicken erythrocytes fell off, while this did not happen with the dogfish erythrocytes, may be explained by the hemolysis which occurred in the former case but not in the latter. In the presence of diphtherial toxins the initial acceleration is followed by a period during which there is no effect, or an inhibition of respiration of all but Arbacia eggs.

It is of interest to note that the rate of oxygen consumption of dogfish erythrocytes is considerably higher than that of chicken erythrocytes. Also, the sensitivity of the respiration of both types of cells to formalin suggests future experiments to study the respiratory mechanisms of these cells.

The relative resistance of the dogfish erythrocytes to the toxins containing lipid-splitting enzymes is worth noting. One of the most outstanding features of the action of toxins on the surface of chicken erythrocytes was the fact that the lecithinase in the toxins of *Cl. perfringens* and *B. cereus* and the lipase in the toxins of *M. aureus* markedly altered the chicken erythrocytes in a very short period of time. Much longer periods of exposure were required to alter the dogfish erythrocytes, particularly in the case of *M. aureus.*

Summary

1. The toxins obtained from *Micrococcus aureus, Clostridium tetani* and *Corynebacterium diphtheriae* accelerate the rate of oxygen consumption of dogfish erythrocytes initially.

2. The toxins obtained from *Streptococcus pyogenes* markedly inhibit the rate of oxygen consumption of these cells after approximately one hour's exposure.

3. The toxins obtained from *Clostridium perfringens, Bacillus cereus* and *Clostridium septicum* have little effect on the oxygen consumption of dogfish erythrocytes.

4. The time for hemolysis of dogfish erythrocytes placed in 0.95 M ethylene glycol is decreased by exposure to the toxins of *Streptococcus pyogenes, Clostridium perfringens* and *Bacillus cereus.*

5. There is a suggestion that the toxins of *Cl. tetani* have a similar effect, but formalized tetanal toxins also decrease hemolysis times.

6. The time for hemolysis of dogfish erythrocytes placed in 0.95 M ethylene glycol is not altered by the presence of the toxins of *Clostridium septicum, Corynebacterium diphtheriae* or *Micrococcus aureus.*

7. The toxins of *Clostridium tetani, Micrococcus aureus* and *Corynebacterium diphtheriae* increase the rate of oxygen consumption of both Arbacia and Asterias eggs.

8. The toxins of *Clostridium perfringens* increase the rate of oxygen consumption of Asterias eggs but have little effect on the respiration of Arbacia eggs.

9. The toxins of *Streptococcus pyogenes* decrease the rate of respiration of Asterias eggs but have little effect on Arbacia eggs.

10. The toxins of *Bacillus cereus* have little influence on the respiration of either Arbacia or Asterias eggs.

11. The toxins of *Clostridium septicum* inhibit the respiration of Asterias eggs but have little influence on Arbacia eggs.

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RESPIRATION AND WATER LOSS IN THE ADULT BLOWFLY, PHORMIA REGINA, AND THEIR RELATION TO THE PHYSIOLOGICAL ACTION OF DDT

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I. Introduction

The interrelations of respiration, muscular activity, water content and content of respirable and irrespirable solid material have been little investigated in insects, particularly at different metabolic rates. The discovery that DDT enhances weight loss and oxygen consumption (Laug, 1945; Ludwig, 1946; Läuger et al., 1946; Buck and Keister, 1946, 1947) makes this insecticide a possible tool for such studies. At the same time, opportunity is afforded for further investigation of the still obscure physiological action of DDT.

II. Materials and Methods

Unless otherwise noted, two- to four-day old adult males of the bluebottle fly, *Phormia regina* Meigen, were used. Larvae were grown on horse meat, and adults given only sucrose, water and fresh orange *ad libitum*. All experiments were done at 25° C. In handling flies, brief carbon dioxide narcosis was used (Williams, 1946). This also reduced feeding variations by causing regurgitation of crop contents (up to 8 per cent of live weight). Flies were poisoned by walking for 10 or 15 minutes in a 1-liter bottle containing 250 mg. DDT deposited as a thick, continuous, tenacious microcrystalline layer which formed slowly from minute supersaturated droplets left after the solvent (5 cc. anesthesia ether) was blown out by 2 minutes' exposure to a strong air current. All doses used were lethal.

For weight-loss experiments, the flies were narcotized, sexed, sorted into four equal groups of 25 to 50, and weighed in cylindrical wire cages, 1 × 2 inches. Two groups were then poisoned with DDT. One poisoned and one control group were set up over 10 per cent NaOH ("Wet Control" and "Wet DDT"), and the remaining two over NaOH pellets ("Dry Control" and "Dry DDT"), in the jar chambers illustrated in Figure 1. The vapor pressure of water was about 19 mm. of Hg over the solution and about 1 mm. over the pellets. Since carbon dioxide was completely absorbed in both types of chamber, conditions were similar to those in the Warburg flasks (see below). Set-up time was about 15 minutes for controls and 32 minutes for poisoned groups. Additional flies were killed with cyanide vapor at the start of some experiments and dried to constant weight at 55–60° C. for initial dry weight percentage.

All four groups were without food, and ostensibly without drinking water, during all experiments, and incoördination made ingestion impossible for the poisoned flies in any case. However, the Wet Control flies might have obtained some drink-

ing water if condensate appeared on the jar walls due to slight environmental temperature changes.

In the three types of weight loss experiments, flies were: (1) Removed briefly at intervals, weighed and replaced, thus giving the rate of gross weight loss. (2) Left for 10 hours, weighed, killed in cyanide vapor and dried to constant weight, giving the loss of dry weight (substrate) in 10 hours. (3) Left until dead, weighed, and dried to constant weight, giving the loss of solid matter previous to death.

Respiration was measured in 15–19 cc. Warburg flasks, one fly to a flask. High and low humidity were obtained by using, respectively, 0.33 cc. 8 per cent KOH, and solid KOH pellets as absorbents for carbon dioxide. The absorptive capacity of the moist surface of the pellets (240 mm^2) was at least as great as that of the 15 by 30 mm. ammonium-free filter paper fan in the "wet" flasks, and the risk of creep-

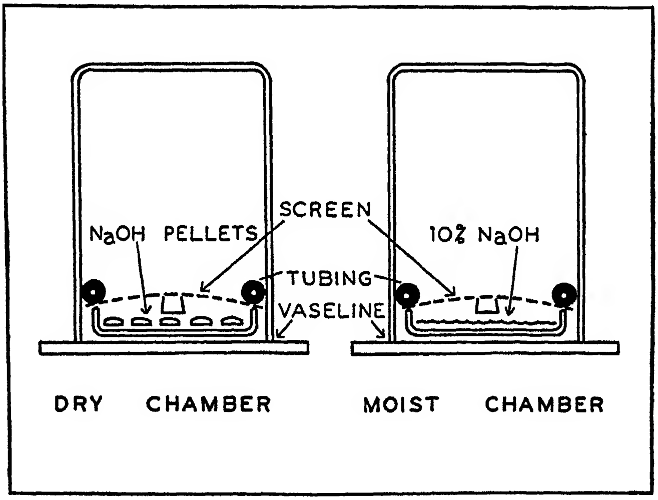

FIGURE 1. Gallon battery jars for use in following weight loss in dry and humid conditions. Flies' (upper) section separated from humidity-control section by circle of window-screen cut slightly larger than inside diameter of jar, and held between ring of pressure tubing and rim of 15 cm. petri dish. Handles attached to centers of screens.

age was much less than with a comparable solution (120 per cent). Flies were segregated from the alkali by cylindrical screens of 50 mesh brass wire. As shown in Figure 2 the paper fan and pellets were kept from touching the screen, since contact of the brass with alkali produced an additional oxygen uptake. Removal of screens when apparent oxygen uptake was maximal showed that they did not significantly impede gas diffusion. In most experiments 10 poisoned and 3 control flies were used. They were weighed individually (while narcotized) to 0.1 mg. just before transfer to the flasks. Each flask was flushed with about 1400 cc. of tank oxygen, which had no consistent effect on uptake or behavior of either control or poisoned flies. Oxygen uptake and individual behavior were recorded every 15 minutes for 6 to 12 hours, then the flies were weighed, killed in cyanide vapor, and dried. Set-up time was 10 minutes for the controls, 25 for the poisoned flies. All

gas volumes are expressed at S.T.P. Dry weights used in computing Q_{O_2}'s (mm^3/mg. dry wt./hr.) were taken as 35 per cent of initial live weights. Evidence validating various technical aspects of our respiration measurements is given in the Appendix.

Although it is usually assumed that carbon dioxide is the only gas liberated by insects, we ran six experiments (52 poisoned flies) in which half the flasks had 0.33 cc. of 5.5 per cent H_2SO_4 in the sidearms, in addition to alkali in the inset. The results indicated that no significant amounts of ammonia were produced by either control or poisoned flies in 5 hours. Likewise, in a number of experiments we interchanged flies between the "acid" and "non-acid" vessels without materially altering the apparent oxygen uptakes (Table IIB).

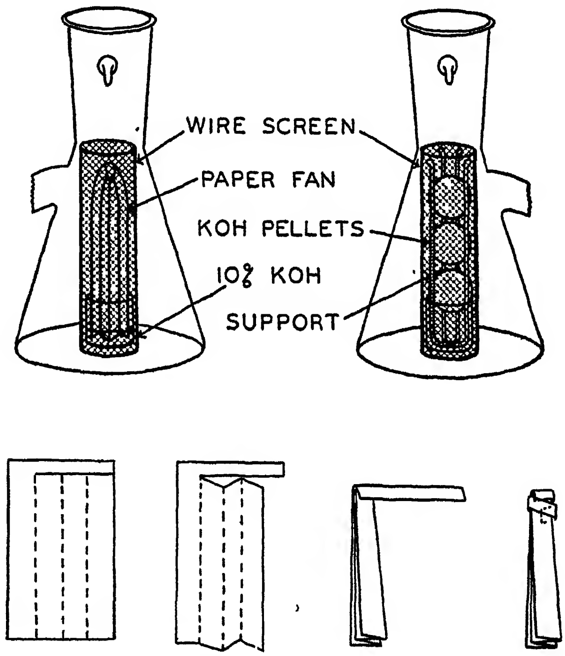

FIGURE 2. Warburg flasks modified for measuring oxygen uptake of flies under dry and humid conditions. The wires of the screening were actually horizontal and vertical rather than diagonal pellet supports made from glass rod. Other explanation in text.

Chitin was estimated by soaking weighed, hemisected flies in 8 per cent KOH for two to three weeks at room temperature, washing thoroughly, filtering on weighed paper, drying and weighing. This does not measure total skeletal material, since a large proportion of many insect cuticles is protein.

The individual variability encountered throughout the work, examples of which are seen in Tables II and III and in the standard errors indicated in Figures 4 and 9, made statistical validation of all conclusions desirable. Probability (P) values for the significance of differences between means were computed by "Student's" t test. The variability was presumably due to variations in age, feeding

and dosage, though it was not materially reduced by applying the DDT in measured individual doses in kerosene solution. Coefficients of variation in weight within groups of 13 flies from a given batch of pupae were very reasonable (6–12 per cent), and an analysis of variance showed that, with flies of comparable age, variation between experiments was no greater than within. Tests of the influence of age and of narcosis time are described later.

III. Results

A. Weight changes in live flies

Weight loss was roughly linear in all four groups during the first 10 hours (Fig. 3) although there was a three-fold difference between the maximum and

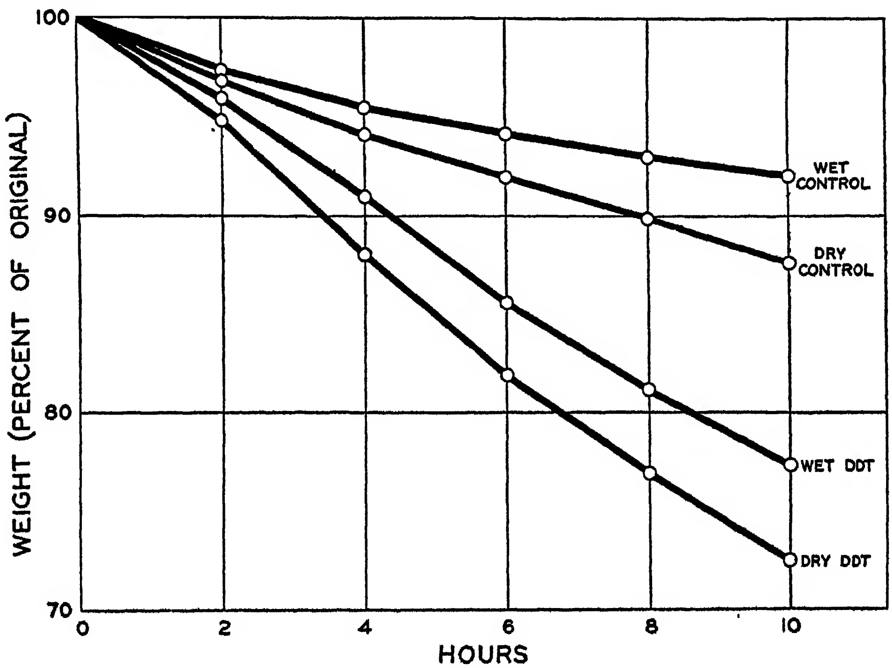

Figure 3. Live weight losses of normal and DDT-poisoned flies under dry and humid conditions. Each point represents the mean weight of 140 flies (6 experiments).

minimum group rates. Both groups in low humidity lost more weight than corresponding groups in high. The fact that the poisoned flies lost more than corresponding controls shows that DDT enhances weight loss. Overall weight loss after 10 hours becomes increasingly uncertain in poisoned groups, since some flies die, but it may considerably exceed the 30 per cent maximum found in 10 hours. In certain control groups, losses of 43 per cent occurred before death. Components of weight loss will be considered with the data on respiration and dry weight.

B. Rate of oxygen uptake of control flies

The oxygen consumption of individuals at rest was about 5 mm^3/mg. dry weight/hour (Fig. 4), a rate comparable to that in several other insects. However, occasional controls showed periods of uptake as great as those of poisoned flies (i.e. up to at least 10 times the usual control rate) coincident with periods of walking or running. Perturbations due to a single such individual out of twelve are seen in the Dry Control rate in Figure 4 (see also Fig. 7), which is otherwise not significantly different from that of the Wet Controls.

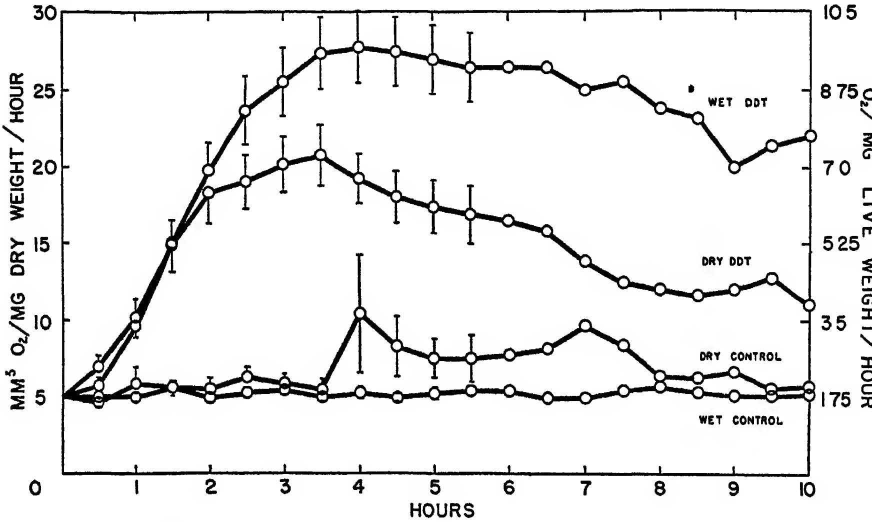

FIGURE 4. Oxygen uptake in normal and DDT-poisoned flies under dry and humid conditions (mean of representative series of 6 experiments). Thirty flies in each poisoned group, 12 dry controls, 6 wet controls. Cap lines from points indicate standard errors. The numbers of flies represented are 78 through 8 hours, 65 at 8½ hours, 51 at 9½ hours, and 35 at 10 hours.

C. Effect of DDT on oxygen uptake

The mean uptake of poisoned flies in a humid atmosphere rose steeply in about 3½ hours to over five times the control value, leveled off for about 3 hours, then declined gradually (Fig. 4). Some individuals showed brief early peaks 15–20 times the control level. The "dry" poisoned flies followed the same course for about 2 hours, but thereafter had very significantly lower uptakes ($P < 0.01$) whether calculated from initial weights or estimated weights at the times of measurement. That this is a true metabolic difference is indicated by the dry weight data given below (Fig. 5 and Table I) and by evidence (see Appendix) that oxygen uptake was measured with equal accuracy under wet and dry conditions. Accordingly, it can be concluded that a dry atmosphere partly inhibits oxygen uptake in poisoned flies. A similar inhibition has been reported in certain normal insects, but is there usually attributable to behavior differences. The unlikelihood that the effect is due to body temperature differences, such as have been observed in normal

insects at different humidities, is indicated by the lack of effect in controls. Probably the explanation lies in the rapid depletion of body water in the "dry" poisoned flies.

D. Relation between oxygen uptake and weight loss

Figure 5 shows the relations between final live and dry weights (as percentages of original live weight) and total oxygen consumptions of individual normal and poisoned flies under dry and humid conditions. The individual variability, reflected in the remarkable spread in total uptakes, is not explained by the differing durations of the six experiments in this series (8½, 8½, 10, 10½, 11 and 12 hours).

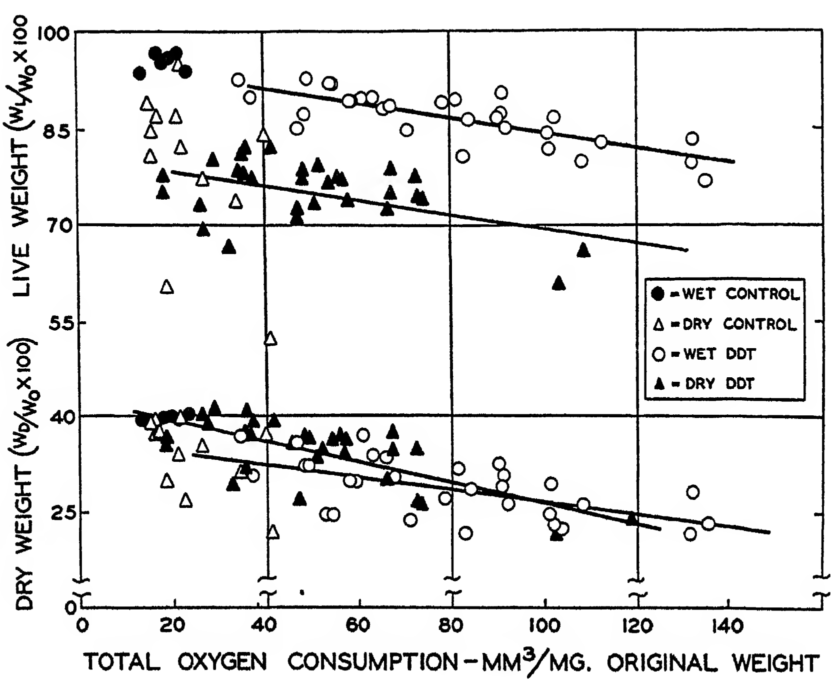

FIGURE 5. Relations between final live and dry weights (as percentages of original live weight) and total oxygen consumption in individual normal and DDT-poisoned flies under dry and humid conditions. Same experiments as those used in Figure 4. Lines fitted by method of least squares.

From Figure 5 we make the following deductions: (1) Loss of both live weight and dry weight of poisoned flies is roughly proportional to oxygen consumption. (2) The proportion of water to total solids changes little in the Wet DDT group. This means, however, that the respirable fraction of the total solid content decreases at a much higher rate than does water. (3) Total oxygen consumption is reduced by dry conditions. (4) Low humidity increases live weight loss. (5) Dry Controls seem to show a greater weight (water) loss in proportion to oxygen consumption than any other group.

E. Respiratory quotient

In experiments in which the insets of half the flasks contained 5.5 per cent H_2SO_4 instead of alkali, the net decrease in gas volume in the flasks with no absorption of carbon dioxide was almost negligible with both control and poisoned flies (Fig. 6 and Table IIA). The R.Q.'s calculated for the plateau period between 3 and 5½ hours were 0.90 for the control flies at rest, 0.93 for all controls, and 0.96 for poisoned flies. The figures indicate oxidation of a largely carbohydrate fuel. The situation is apparently different in poisoned Japanese beetle larvae, where Ludwig, by an undescribed method, obtain R.Q.'s of 0.6 to 0.8 and showed by analysis that much fat was metabolized.

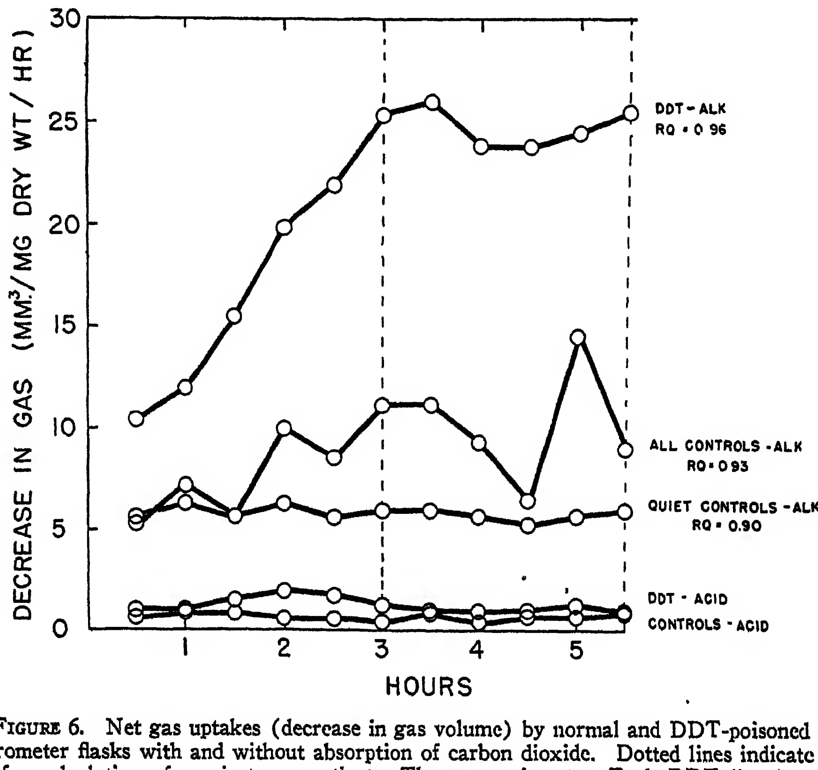

FIGURE 6. Net gas uptakes (decrease in gas volume) by normal and DDT-poisoned flies in respirometer flasks with and without absorption of carbon dioxide. Dotted lines indicate period used for calculation of respiratory quotient. Three experiments. Each DDT line based on 8 flies; alkali controls at rest, 3; all alkali controls, 6; acid controls, 9 (6 of which were at rest). Figures on 10 additional poisoned flies are given in Table IIA. The disturbing effect of activity on uptake is illustrated in the graph for all the alkaline controls.

F. Components and mechanism of weight loss

Since weight loss is nearly linear over the first 10 hours and is proportional to total oxygen consumption during the same period, rate of loss would be expected to be proportional to rate of oxygen uptake. However, uptake is far from linear in the poisoned flies, indicating that a breakdown of overall weight loss is desirable.

In Figure 7, the dotted line running through all the columns indicates the mean initial total solids (35 per cent).[1] The supposedly "irrespirable" fraction (11.9 per cent) is defined as the dry weight of Wet Control flies at death from starvation (minus the 2.5 per cent chitin fraction). The value for the "respirable solids", or material which can be used up in metabolism (20.6 per cent), was obtained by subtracting the weights of chitin and irrespirable solids from the total dry weight.

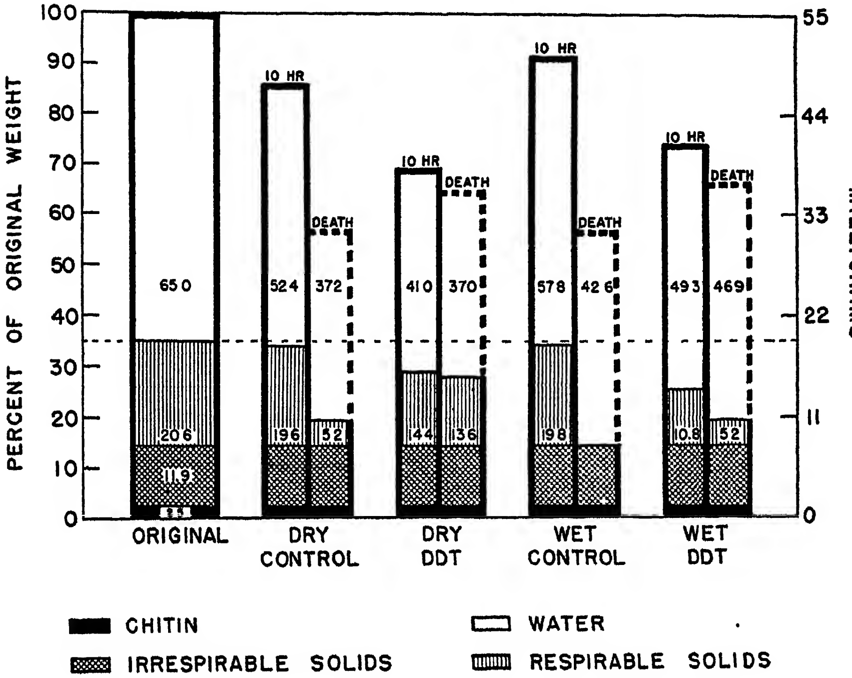

Figure 7. Content of water, respirable and irrespirable solids and chitin at 10 hours and at death of normal and DDT-poisoned flies in dry and humid conditions as percentages of original live weights. Based on measurements on about 860 flies in 9 experiments, including the 560 flies used for rate of weight loss measurements (Fig. 3). Water content at death subject to some uncertainty because flies did not all die simultaneously, and because the time of actual death was uncertain.

Figure 7 shows that live weights at 10 hours were in the order: Wet Control > Dry Control > Wet DDT > Dry DDT (in per cent of original live weights, 92.0 ± 1.30; 86.4 ± 1.42; 74.5 ± 1.39; 69.8 ± 1.92). All groups differ significantly ($P < 0.01$) except the Wet DDT and Dry DDT ($P = 0.08$). This indicates that both a dry environment and DDT enhance weight loss. Dry weights at 10 hours were in the order: Wet Control and Dry Control > Dry DDT > Wet

[1] This figure increases slightly with age. This explains the fact that at presumptive zero oxygen uptake, the dry weights of the flies used in the wet-dry respiration experiments (Fig. 5) average nearer 40 per cent than 35, since all the flies in this eight-day series of experiments came from the same batch of pupae.

DDT (34.2 ± 1.47; 34.0 ± 1.30; 28.8 ± 0.64; 25.2 ± 0.33), the poisoned flies having reversed their relative positions. All groups differ significantly except the controls. The data agree with the respiration experiments in indicating that DDT enhances respiration (substrate loss), and that a dry atmosphere reduces this stimulation. Furthermore, they show that DDT promotes both water loss and substrate loss though not proportionately, and that water loss is greatest in the Dry DDT flies.

TABLE I

Interrelations of weight loss and oxygen consumption at 10 hours. Total oxygen uptakes (Col. 1) were estimated by graphical integration of the areas under the curves in Figure 4. Values in Columns 2, 3 and 5 come from the weight loss experiments (Fig. 6). Dry weights estimated from oxygen uptakes (Col. 4) were calculated on the basis of glucose, as were the weights of metabolic water corresponding to oxygen uptakes (Col. 7) and observed dry weight losses (Col. 8). Reserve water (Col. 9) was obtained by subtracting the means of Columns 7 and 8 from Column 3. "Metabolic water" in Column 10 is the mean of values in Columns 7 and 8.

	Mean total O_2 uptake/fly (mm^3)	Mean total weight loss/fly (mg.)	Mean total water loss/fly (mg.)	Mean total dry weight loss/fly (mg.)		Per cent water in total loss	Metabolic water loss (mg.) calculated from		Reserve water loss (mg.)	Per cent metabolic water in total water loss
				Calc.	Obs.		O_2 uptake	Obs. dry wt. loss		
	1	2	3	4	5	6	7	8	9	10
Wet DDT	4300	14.0	8.6	5.8	5.4	61	3.5	3.2	5.2	39
Dry DDT	2820	16.6	13.2	3.8	3.4	80	2.3	2.1	11.0	17
Dry Control	1300	7.5	6.9	1.7	0.6	92	1.0	0.36	6.2	10
Wet Control	960	4.4	4.0	1.3	0.4	91	0.8	0.24	3.5	13

Since, with the possible exception of the Wet Controls, the flies do not drink, it is possible to estimate the contributions of "metabolic" and "reserve" water to total water loss, on the basis of the observed R.Q. of nearly 1. As Table I shows, metabolic water estimated from oxygen uptake and that estimated from dry weight loss agree surprisingly well for the poisoned flies, considering the crudeness of the data and the fact that they were based on quite different sorts of experiments. The estimates for the controls are less reliable because of fewer flies in the respiration experiments and proportionately larger arithmetic errors in computing dry weight losses. However, the higher observed dry weight loss in the Dry Controls is consistent with the observation that this group was more active than the Wet Controls. Table I shows also that DDT increases both reserve and metabolic water losses, and that the former was several times as great as the latter. This fact plus the fact that water accounts for most of the weight lost (Col. 6) explain why the weight loss rates of the poisoned flies (Fig. 3) showed only a slight acceleration between 2 and 6 hours, when respiration increased several-fold.

Since poisoned flies did not defecate (see section on behavior), evaporation was the only significant avenue of water loss. The increased rate of loss might be associated with an increase in the normally low cuticular permeability but seems more likely to be due simply to increased transpiration, such as would occur if DDT

caused the spiracles to remain open abnormally long. This would be consistent with the neuromuscular action of DDT, and also with the augmented metabolic rate. Unfortunately the abdominal spiracles of Phormia are too minute, and the great thoracic spiracles too inaccessible, for study without narcosis or artificial restraint. However, in the firefly *Photinus pyralis,* where the abdominal spiracles can be observed under relatively normal conditions, they open permanently in the stage of poisoning which corresponds symptomatically with the period of increasing respiration in Phormia (Buck, 1948).

Death occurred in less than one day in the average Dry DDT fly and more than eight in the Wet Controls. The water content of both Dry Controls and Dry DDT flies was around 36 per cent at death. However, the Wet DDT group still contained about 47 per cent water at death, hence death from DDT cannot be ascribed primarily to water loss.

As shown in Figure 7, the dry weights of flies which remained in the chambers until death were in the order: Dry DDT > Dry Control and Wet DDT > Wet Control (in per cent of original live weights, 28.0 ± 1.00; 19.6 ± 0.69; 19.6 ± 0.69; 14.4 ± 0.42). All groups differ very significantly except the Dry Control and Wet DDT. The Wet Control thus showed the lowest weight, instead of the highest as it did at 10 hours, and the Dry DDT the highest, instead of the next to lowest as it did at 10 hours. Hence, poisoned flies, although respiring faster than controls early in the experiment, died before they had lost as much solid material. Consequently, general substrate exhaustion cannot be the primary cause of death, assuming that poisoned flies utilize the same materials as controls.

G. Influence of hypoxia on DDT poisoning

In poisoned *Musca domestica* in air, Laug observed that oxygen uptake attained a high rate in 2 hours, then fell off rapidly to zero, coincident with cessation of movement in the fly. We observed the same thing in Phormia. Calculation showed that the cessation of uptake coincided with the exhaustion of oxygen in the flasks. Thus when the flasks were flushed with air or oxygen, the flies resumed kicking, and oxygen consumption again rose (Fig. 8). Therefore hypoxia, like cyclopropane anesthesia (Merril, Savit and Tobias, 1946), delays but does not permanently inhibit the symptoms. The symmetry of the initial peak (Fig. 8) indicates that pO_2 becomes limiting at about 90 mm. (10 per cent). This concentration was found limiting to oxygen uptake in flying *Lucilia sericata* by Davis and Fraenkel (1940), and to wing-beat frequency of flying Drosophila by Chadwick and Williams (unpublished).

In flies reviving from hypoxia, oxygen uptake often exceeded the original peak (Fig. 8), but the total uptake in the second burst of respiration showed little if any increase over the first. Since renewal of alkali (and air) after the second burst did not raise oxygen uptake above the control level, the total of the two bursts (ca. 6000 mm^3) either represented the maximum uptake of which those individuals were capable or indicated that no marked oxygen debt had been incurred.

H. Dosage effect

Laug found that house flies given 2.5 μg DDT/fly showed an earlier and smaller increase in oxygen uptake than flies given 1 μg/fly. Similarly, Ludwig's graphs indicate that peak uptake following 5 per cent DDT was higher than with either 10

per cent or 1 per cent, and that total uptakes were in the order 1 per cent > 5 per cent > 10 per cent.

To evaluate dosage effects in Phormia, oxygen uptakes were measured in culture-mates given 10 minute and 30 minute exposures to a DDT surface. As Figure 9 shows, oxygen consumption rose equally in the two groups to about three

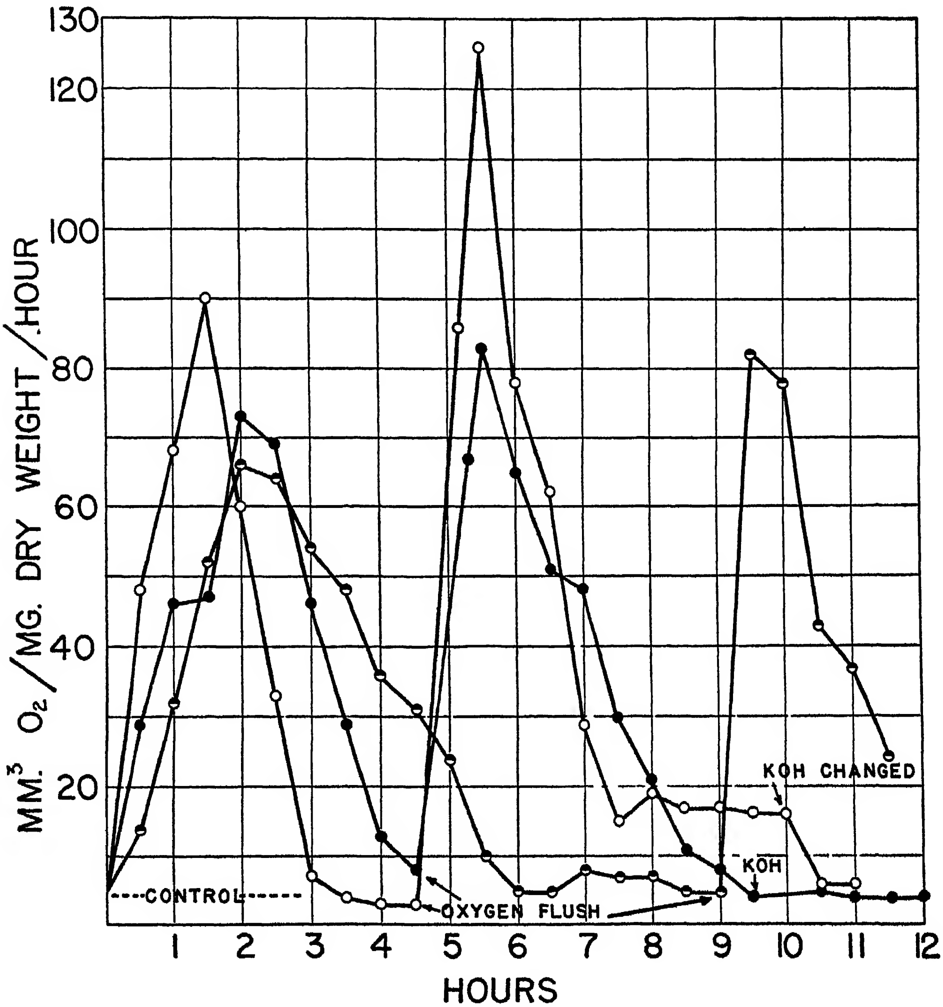

FIGURE 8. Oxygen uptakes of 3 flies respiring in air in Warburg vessels of about 15 cc. volume. Vessels flushed with oxygen at indicated points, and alkali renewed in 2 at indicated points.

times the control rate. Thereafter the more heavily dosed flies showed the lower uptake, the difference being highly significant ($P < 0.01$) after 3 hours. Therefore, since heavier doses ordinarily give a quicker and larger kill than lighter, excessive respiration is not the direct cause of death from DDT.

I. Behavior of poisoned flies

The visible symptoms of DDT poisoning, though non-specific and individually variable, are of interest in connection with the origin of the enhanced oxygen uptake. Three indistinctly separated stages of poisoning could usually be recognized. The first, lasting 15 to 30 minutes, involves locomotor hyperactivity with progressive incoordination ending in prostration. In the second, lasting ½ to 1½ hours, the flies lie on their backs, exhibiting violent spasmodic extensions and flexions of the legs, with irregular movements of proboscis and wings. Occasionally the wing movements go over into a burst of furious buzzing which lasts a minute or more.

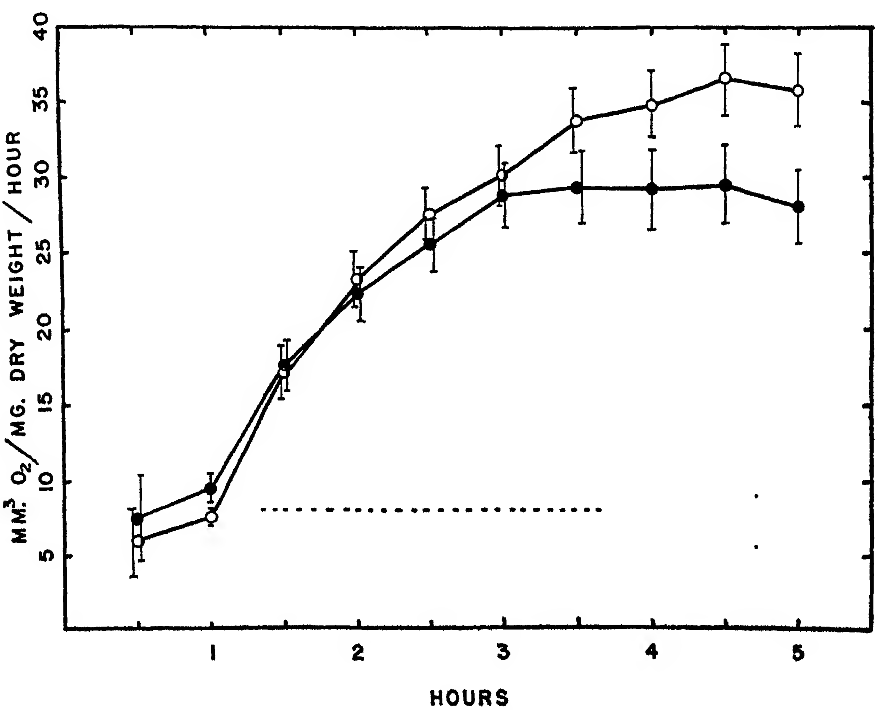

FIGURE 9. Oxygen uptake in flies given 10 minutes' exposure to DDT (open circles) and 30 minutes (closed circles). Cap lines on points indicate standard errors. Dotted line indicates control level. Upper curve 52 flies, lower curve 47 (11 experiments).

The third stage involves marked high-frequency tremors. The legs become more or less constantly flexed at the femoro-tibial joints, so that all six tarsi are brought close together. This stage continues for several hours, during which the tremors become less and less vigorous, and finally can be seen only under the microscope. At this point the fly usually voids a large drop of excrement (up to 4 mg.), the first since the start of the experiment. This adheres to the ventral surface of the abdomen and is included in the weight.

It is difficult to conclude whether or not the overt muscular activity corresponds well with the rate of oxygen uptake, although it would not necessarily do so if some

muscles were in tetany. In accord with Laug's finding, prostration occurs before peak oxygen uptake is attained. Except for the brief periods of buzzing, when very high uptakes were recorded, the first two stages are usually past before peak uptake is reached. This is somewhat surprising because the kicking of Stage 2 appears to be the most violent sustained motor activity during poisoning. However, the gradually decreasing tremors during Stage 3 correspond well with the prolonged "plateau" period of oxygen uptake (Fig. 4), and the terminal, incontinent phase, where visible activity is minimal, is always associated with a very low uptake. Correlation of activity with uptake is also indicated by the fact that the flies given a 30 minute exposure to DDT in the dosage experiments showed less violent symptoms than those with a 10 minute exposure, and appeared to die sooner.

J. Hydrogen ion concentration in DDT poisoning

Another possible factor in the toxicity of DDT would be an acidosis from metabolic products accumulated during hyperactivity. We investigated this question crudely on breis made by blending groups of 50 flies in 35 cc. of distilled water for 2 minutes. Glass-electrode determinations on control groups kept 12 hours without food or water gave pH values of 7.00, 7.03 and 7.00. Similar determinations on poisoned flies, at a time when one-half to two-thirds of them had ceased motion (12 to 16 hours), gave pH values of 7.04, 7.13 and 7.13. All values are within the range of the bloods of many insects (Boche and Buck, 1942). The data indicate that there is no generalized acidosis during DDT poisoning.

K. Age and narcosis time as variables affecting the response to DDT

Numerous workers have reported that newly hatched or young insects are more susceptible to poisons than older insects, although the reverse seems to be true in some instances. To test the age factor, oxygen uptakes were measured in five-fly samples from the same batch of pupae at 4 and 8 days of age, and in nine-fly samples from two different batches (average ages 2 and 12 days). The results indicated clearly an earlier response and greater rate and total of oxygen uptake in the younger flies. This is somewhat unexpected in view of the finding of Williams, Barness and Sawyer (1943) that the glycogen content (and hence presumptive respiratory capacity) of Drosophila rises steeply during the first 7 to 10 days of adult life.

Oxygen uptakes of groups of 15 flies given 10 and 35 minutes' narcosis before poisoning were identical over 6½ hours. This indicates that the unavoidable slight differences in narcosis time in our experiments were unimportant. However, in flies exposed for 160 minutes to carbon dioxide before poisoning, the subsequent uptake was only slightly above the usual control level, and less than one-third that of companion flies given only 5 minutes' narcosis.

IV. Discussion

From the observations that anesthetics postpone the carbohydrate depletion (Merril, Savit and Tobias), the increased oxygen uptake (Läuger et al.), and the symptoms (Bodenstein, 1946) of DDT poisoning, it has been concluded that the increased uptake is due to the increased motor activity rather than to a specific stimulation of oxidative metabolism. This view is supported by our observations

of a rough correlation between degree of activity and rate of oxygen uptake, and of high uptakes in active controls. Further evidence is afforded by the parallel between some of the physiological changes during poisoning and those during normal flight (for review see Chadwick and Gilmour, 1940; Williams, Barness and Sawyer; and Chadwick, 1947). Points of similarity are: (a) marked carbohydrate depletion, (b) R. Q. of about 1, (c) ten- to twenty-fold peak increase over resting oxygen uptake, (d) no well-defined oxygen debt, (e) oxygen becomes limiting to maximal activity at a tension of about 90 mm.

Ludwig's conclusion that carbohydrate exhaustion is the direct cause of death in poisoned Japanese beetles is opposed by the evidence of Merril, Savit and Tobias that roaches are not saved by glucose administration, nor by anesthesia sufficient to suppress symptoms and prevent carbohydrate depletion; by Chadwick's conclusion that normal Drosophila can survive for some hours after their carbohydrate has been exhausted; and by the present finding that flies dead from DDT poisoning have more "respirable substrate" left than starved controls.

V. Summary and Conclusions

Weight, oxygen uptake, and behavior were followed in normal and DDT-poisoned adults of the bluebottle fly, *Phormia regina*, under very humid and very dry conditions, with the following results:

1. Live weight loss was roughly linear over at least the first 10 hours. Poisoned flies lost more weight than unpoisoned, and "dry" groups more than corresponding "wet" groups. Water formed about 60 per cent of total weight loss in "Wet DDT" flies, and from 80 to 90 per cent in the other groups, and was probably lost almost entirely by spiracular transpiration. Metabolic water loss calculated from total oxygen uptakes was in reasonably good agreement with that calculated from dry weight loss. It is concluded that both DDT and a dry atmosphere enhance water loss; that DDT enhances loss of both metabolic and reserve water; and that loss of water is not the primary cause of death in DDT poisoning.

2. DDT induced an average five-fold increase in oxygen uptake of flies in a moist atmosphere. Individuals reached transient peaks of 15 to 20 times the control rate, associated with violent wing buzzing. The increase was significantly smaller in a dry atmosphere.

3. At the end of 10 hours, the dry weights of the four groups were in the order: Controls > Dry DDT > Wet DDT. At death the order was: Dry DDT > Dry Control and Wet DDT > Wet Control. It is therefore concluded that death is not primarily due to exhaustion of respirable substrate, assuming that the poisoned flies utilize the same materials as the controls. This conclusion is also supported by the fact that a heavier, more toxic dose of DDT produced a smaller increase in oxygen uptake than did a lighter.

4. Over a 10 hour period total oxygen uptake was proportional to both live and dry weight losses in the poisoned flies. In the Wet DDT flies, relative solid content remained approximately constant, and respirable substrate decreased at a higher rate than did water.

5. The estimated respiratory quotient was about 0.90 in controls, 0.96 in poisoned flies. Oxygen became limiting to the enhanced uptake, and to hyperactivity, at a tension of about 90 mm. Active controls reached rates of oxygen uptake com-

parable to those of poisoned flies. Overt activity showed a rough correlation with rate of oxygen uptake. Several parallels between metabolism in normal flight and physiological manifestations of DDT poisoning are pointed out. It is concluded that the increased oxygen uptake in DDT poisoning is due to the motor hyperactivity induced.

6. No ammonia production was found in either control or poisoned flies.

7. Hydrogen ion determinations on breis of normal and poisoned flies indicated that no general acidosis occurred during DDT poisoning.

8. The roles of dosage, age and narcosis in the variability of the results are discussed.

We are happy to acknowledge the advice of Drs. Edward Adolph, Leigh Chadwick, Edwin P. Laug, Kenneth Roeder, A. Glenn Richards, Julian Tobias and J. Franklin Yeager.

Appendix

Validative data on technique of respiration measurements, with tables concerning respiratory quotient, ammonia production and variability

1. Measurement of oxygen uptake at different humidities. Since oxygen uptake in Warburg flasks is almost always measured under humid conditions, it is necessary to show that no systematic physical error was involved in our measurements in a dry atmosphere. Calculation shows that the maximum possible error from the diverse ratios of water vapor to gas at the two humidities (which might produce a spurious excess in uptake in the more humid flask) is negligible. Heat of solution of the alkali pellets is likewise unimportant because no appreciable difference was seen in the apparent rates of oxygen uptake of dry and wet flies during the first two hours, when the effect should have been most pronounced, and no disturbance occurred in the Dry Control rate when moist outside air was introduced in resetting the manometers.

As an overall empirical check on our technique, we exchanged "wet" and "dry" flies at times when their rates of oxygen uptake were apparently steady (from 2¼ to 6½ hours after poisoning). In 25 flies transferred from "wet" to "dry" flasks, the mean rate of uptake in the half hour immediately following the change was 98.6 ± 4.0 per cent of that in the corresponding period previous to transfer. In 25 "dry" to "wet" transfers, the corresponding new rate was 98.3 ± 7.2 per cent of that prior to transfer. Similar results were obtained in comparing the hour previous to transfer with the hour subsequent to transfer. (The fact that uptake decreased after both exchanges merely reflects the usual gradual fall with time.) These results strongly indicate that our technique measured oxygen uptake with equal accuracy in high and low humidity, and that the difference in uptake at the two humidities is metabolic. Furthermore, it appears that the humidity factor, whatever its ultimate cause, operates only before the uptake plateau is attained.

2. Linearity and capacity of carbon dioxide absorption. In some experiments, apparent oxygen uptakes of over 2000 mm^3 per hour per 55 mg. fly were recorded for brief periods, and even the average plateau level for all the Wet DDT flies was over 500 mm^3/hr./fly (multiply the mm^3/mg. dry wt./hr. values given in the figures and tables by 19). Since these rates, and the total uptakes per fly over 8 to 10 hours, are far higher than those usually measured with the Warburg microrespirometer, it is necessary to show that absorption of carbon dioxide was not limiting in our experiments. In most of 12 instances in which two flies were put together after they had apparently reached their plateau phases of uptake (Table III) the resulting apparent oxygen uptake in the single vessel closely approximated the sum of the two previous individual uptakes (rates up to nearly 3500 mm^3/hr. were obtained). It consequently seems clear that the absorptive capacity of the alkali was adequate, as far as rate of production of carbon dioxide is concerned, and that the plateau period of uptake (Fig. 4) is not an artifact.

The calculated total carbon dioxide capacity of the alkali in the inset of the average flask was about 5300 mm^3. Although in the longest experiments (Fig. 4) the average total oxygen

uptake was only about 4300 mm³ in the most active group, several individuals approached, and (on the basis of an R. Q. of 1) a few even apparently exceeded the theoretical limit of carbon dioxide absorption. Accordingly, unless the R. Q. decreased late in the experiments (as might have happened, in view of Chadwick's finding of a great decrease in R. Q. of Drosophila after flight), it is possible that the oxygen consumptions recorded in the last hour or two of some of the experiments were actually somewhat lower than the true values. In a few flasks in which the alkali was renewed, uptake increased, but in others (for example some of those shown in Fig. 8 and Table III), it did not. Thus some individuals show a decrease in oxygen uptake like that shown in the average curve in Figure 4, even when there are no known technical limitations

TABLE II

Oxygen uptake in paired individual flies before and after exchanging experimental conditions. Data used in calculating R. Q. and evaluating inhibition by accumulating carbon dioxide (Part A) and in testing ammonia production (Part B). In part B "decrease in gas volume" is equivalent to "oxygen uptake." From 15 to 30 min. were lost in each transfer and re-equilibration.

Expt.	Fly	Original treatment	Rate of decrease in gas volume in successive ½ hr. periods before exchange (mm³/mg. dry wt./hr.)	Time of exchange (hr.)	Rate of decrease in gas volume in successive ½ hr. periods after exchange (mm³/mg. dry wt./hr.)	Rate of decrease in gas volume in successive ½ hr. periods after return to original state (mm³/mg. dry wt./hr.)
A. Exchanges between flasks with CO_2 absorption ("KOH") and those without ("acid")						
166	3	KOH	23 40 56 63	2¾	0.5* 1.3 1.2 0.3	52 31 15 23
	4	Acid	0.5 0.5 0.7 1.1		9 10 12 11	0.1 0.8 0.8 0.1
	5	KOH	33 31 33 33	5	1.1 1.2 1.1	32
	6	Acid	1.0 0.7 0.9 0.7		14 18 21	0.7
	12	KOH	46 38 32 36	3¾	0.6 1.2 1.1 0.8	15 21 30
	13	Acid	1.3 1.5 1.0 1.0		19 18 16 13	0 0.4 0.8
168	3	KOH	16 49 64 70	2½	2.4 2.1	33 15
	6	Acid	1.0 0.9 1.3 1.2		22 25	0.7 0.9 0.9
	5	KOH	29 36 38 41	3	1.0 1.1 0.7	55 62 55
	11	Acid	1.2 1.6 1.5 1.2		41 41 36	0.4 0.7 0.5
B. Exchanges between flasks with and without acid in side arms						
164	6	Acid	31 43 69	2½	37 42	38 36 34 32
	9	Non-acid	22 44 57		87 51	20 15 18
	13	Acid	11 28 53 56	2¾	56 47 44	41 34
	14	Non-acid	16 34 36 26		32 34 30	35 34
165	9	Non-acid	27 22 31 33	4¾	28 23 12	
	14	Acid	36 36 40 42		35 33 27	

Table III

Oxygen uptake of paired individual flies separately and after being combined in one respirometer flask to test linearity of carbon dioxide absorption. Note that the uptakes are rates per unit weight, and thus those in the last column should be expected to approximate the means of the individual uptakes of the two combined flies, rather than their sums. Table also shows variation in "plateau" uptake levels within and between flies, variation in individual responses with time, and equivalence of carbon dioxide absorption by pellets and solution. Expts. 151 and 152, wet-dry comparisons; 162 and 164, testing for NH_3; 170–178, dosage. From 15 to 30 min. were lost in each transfer and re-equilibration.

Exp.	Fly	Original treatment	Rate of O_2 uptake in successive ½ hr. periods before combining (mm³/mg. dry wt./hr.)	Time of combining (hr.)	Rate of O_2 uptake in successive ½ hr. periods after combining (mm³/mg. dry wt./hr.)
151	4	Wet	16 12 11 12	6½	13 14 15 19
	6	Wet	16 15 17 14		
	5	Dry	9 11 14 9	6½	10 7 7 5
	7	Dry	13 14 19 21		
	9	Dry	9 8 8 9	8½	30 15 16
	11	Dry	33 35 38 39		
152	6	Wet	25 23 20 20	5½	19 18 16
	10	Wet	18 19 18 19		
	11	Dry	20 23 16 16	5½	22 10 6 4
	13	Dry	42 35 31 28		
162	2	Non-acid	36 37 29 34	6½	30 33 34
	10	Non-acid	26 29 33 29		
164	2	Non-acid	40 56 46	1¾	27 27 26*
	3	Non-acid	30 47 48		
170	3	10′ DDT	40 40 42 44	5½	46 41
	12	10′ DDT	43 39 42 39		
172	7	10′ DDT	33 42 45 53	5½	46 43†
	12	10′ DDT	40 44 42 43		
173	11	30′ DDT	19 33 45 52	3½	50 45*
	13	30′ DDT	59 69 55 59		
175	9	30′ DDT	49 60 74 74	4½	86 33*
	11	30′ DDT	38 41 46 52		
178	4	10′ DDT	57 44 46 49	7	45*
	6	10′ DDT	76 72 74 79		

* These flies were subsequently separated, and after addition of new KOH and oxygen their individual uptakes were lower than before they were combined.

† Addition of new KOH and oxygen did not change uptake.

on measurement. Adequacy of carbon dioxide absorption is also suggested by the correlation between total uptake and weights of Wet DDT flies (Fig. 5 and Table I).

3. Estimation of respiratory quotient The method of estimating R. Q. depends on average oxygen uptake being substantially the same in the two sets of flies. This requirement would not be met if the activity of the flies in the "acid" vessels was reduced by the accumulating carbon dioxide (calculated potential maximum of 7 per cent in 3½ hours). The possibility of extensive inhibition is minimized by the facts that the net uptake in the acid vessels did not change; that no difference in behavior was observed in the two sets of flies; and that no change was produced in one of the acid vessels by flushing with oxygen. In supplementary exchange experiments (Table IIA), 4 of 5 flies originally in acid flasks did indeed have considerably lower rates of uptake than their partners originally in alkali flasks. However, the R. Q. calculated from the acid and alkali periods of the flies originally in acid flasks is 0.95, which is so close to the 0.96 derived from the data given in Figure 7 that it seems legitimate to conclude that the estimated R. Q.'s are of the correct order of magnitude.

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GROWTH OF OYSTERS, O. VIRGINICA, DURING DIFFERENT MONTHS

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Introduction

Although the American oyster, *Ostrea virginica,* is one of the most common bivalves of our Atlantic and Gulf Coasts, very little attention was given by past workers to various aspects of its growth, despite the fact that this field offers a large number of unanswered problems. For example, Moore (1898) in his voluminous article on the oyster devoted less than one page to a discussion of its growth. Churchill (1921) also confined himself to a few general statements on the growth of oysters under different environmental conditions. Yet, an understanding of the growth of oysters is of undeniable importance not only from a purely biological, but also from a practical, point of view because the oyster industry occupies one of the leading positions among the fisheries of the United States.

Nelson (1922) was perhaps the first to study more or less systematically certain phases of the growth of oysters. He measured and weighed a large group of New Jersey oysters in April and August of 1919 and again in March of 1920 noticing the increase in size and weight between the measurements. Loosanoff (1947) and his associates observed the increase in size of oysters of different ages grown during a three-year period in Milford Harbor, Connecticut. The oysters were measured once a year—in late autumn—to show the increase in size.

None of the observations made thus far was directed to study the relative increase in the size and volume of the oysters during each month of the year. This article presents the results of such studies which were carried on in Milford Harbor, Connecticut.

Winter Observations

Because it has never been satisfactorily shown whether New England oysters continue to grow during the hibernation period, experiments were carried on for three successive winters to give the needed answer.

Oysters were prepared for the experiments as follows: After the shells were cleaned of all foreign matter, the edges of the shells of each oyster were filed off to make it easier to notice new growth if any formed. The oysters were then individually numbered with small celluloid tags. Later the length, width, and depth of each oyster were measured with a vernier caliper reading to 0.1 mm. The length represented the greatest anterior-posterior dimension. The width was measured along the maximum dorso-ventral line, and the depth represented the maximum distance between the outer surfaces of the two shells.

For determining the volume of an oyster a modified Grave's (1912) method, consisting of measuring the quantity of water displaced by an oyster, was used.

The oysters were kept moist before immersion, to avoid a possible error in determining the volume, because dry shells usually absorb small quantities of water. The method was found simple but reliable, the measurements being accurate within one or two per cent.

The first experiment was begun with 80 three-year-old oysters. After the measurements were completed on December 7, 1944, the oysters were put into a large wire tray which was placed on the bottom of Milford Harbor at a depth of approximately 3 feet below the mean low water level. The water temperature on that date was 3.0° C. Soon after the tray was placed in the Harbor a layer of ice was formed and, therefore, the oysters could not be examined at frequent intervals.

The first examination was made on March 7, 1945 when the temperature of the water was still near 0.0° C. Examination of the edges of the shells showed that not in a single case was new growth formed. The final examination was made on March 20, when the water temperature was reaching 5.0° C., thus indicating that the end of the hibernation period was approaching. During this examination the length, width, depth, and volume of each oyster were re-measured and the data compared with those obtained for the same individual the preceding fall. Only three oysters died during the winter. The measurements of the living 77 oysters showed that they did not change in size or volume during the winter.

The second experiment was made during the winter of 1945–1946. A group of 120 oysters was placed in Milford Harbor at the beginning of the hibernation period and re-examined in March. All the oysters survived the winter but their shells did not increase in length, width, or depth.

The third and final experiment was conducted with 58 oysters between December 9, 1946 and March 14, 1947. In addition to the observations made during the two previous winters, the weight of each oyster was ascertained at the beginning and at the end of hibernation. The results of the March measurements showed that with the exception of one oyster which had part of its shell broken off, there was no change in length, width, depth, volume, or weight during the winter.

As a result of the observations made during three winters, we may conclude that in northern waters the shells of the oysters do not increase in size, volume, or weight during the hibernation period. However, our laboratory observations, which will be discussed in a later part of this article, showed that if the temperature of the water is kept well above the hibernation point, the oysters will continue to grow even in the middle of winter.

Observations During the Growing Period

The first experiments to determine the increase in the size and volume of the oysters during each month of the growing period were begun in the spring of 1944, but had to be discontinued in the middle of the summer because the new growth, which is almost as thin as cigarette paper and extremely brittle, broke off at the slightest touch. The experiments started in 1945 were also discontinued several months later for the same reason.

Finally, in 1946 the observations were successfully completed. On March 29, 1946, a group of 120 adult oysters was placed in a large wire tray attached to a float anchored in Milford Harbor. The float rose and fell with the tide but the position of the tray always remained approximately 3 feet below the surface of

the water. Before placing the oysters in the tray they were individually numbered, measured, and their volumes determined.

The oysters were re-examined and re-measured at the end of each month; the last measurements were made during the last days of November when the water temperature was becoming low enough to induce hibernation. To keep the oysters out of the water as little as possible during the examinations they were handled in groups of ten, because such small groups could be measured and returned to the water within a few minutes. Also, by working with small groups it was easier to avoid breaking the shells.

Of the original group of 120 oysters, 109 were alive at the end of the experiment. The conclusions offered in this article are based upon the data obtained from these survivals. The ranges in length, width, depth, and volume of the oysters at the beginning of the observations were 68.2–107.5; 50.3–85.8; 22.5–40.0 mm.; and 40–104 cc. respectively. At the end of the experiments the ranges were 85.3–135.0; 66.5–107.6; 26.4–44.3 mm.; and 65–176 cc. The mean length, width, depth, and volume of the oysters for each month are given in Table I.

TABLE I

Mean with standard error of length, width, depth and volume of oysters at the end of each month during growing period of 1946, Milford Harbor

Month	Mean			
	Length in mm.	Width in mm.	Depth in mm.	Volume in cc.
March	88.2±0.766	65.4±0.519	30.0±0.314	63.7±1.438
April	88.7±0.799	65.6±0.554	30.0±0.319	64.8±1.519
May	93.8±0.823	71.0±0.604	30.0±0.319	65.7±1.518
June	98.1±0.886	79.4±0.766	30.0±0.319	69.0±1.531
July	103.1±1.001	82.5±0.763	31.0±0.309	73.6±1.675
August	106.0±0.95	83.8±0.788	32.1±0.329	84.1±1.893
September	109.6±1.034	86.0±0.786	33.2±0.352	93.0±2.243
October	110.4±1.06	86.3±0.8	34.1±0.37	97.2±2.464
November	110.8±1.084	86.3±0.8	34.6±0.365	99.3±2.596

In estimating the monthly increases of each variate, which represented the difference between the means of each two consecutive months, the total increase for the entire growing season was taken as 100 per cent, and the monthly gains were calculated in relation to it. The results are presented in Figures 1 and 2.

As already mentioned, the oysters of our area do not increase in size or volume during the hibernation period, which extends roughly from the beginning of December until the end of March. Monthly observations showed, however, that in April the shells of the oysters begin to grow, the mean increase in length for that month constituting 2.21 per cent of the total annual increment (Fig. 1). During May, June, and July the increase in length is most rapid, being 22.57, 19.03 and 22.12 per cent respectively. Thus, during these three months the oysters achieved approximately 63.7 per cent of their annual increase in length. The percentages for August, September, October and November were 12.83, 15.93, 3.54 and 1.77

respectively. As can be seen, the months of October and November contribute but little to the total annual increase in length.

An increase in the width of the oyster shells began in April, simultaneously with an increase in length, but terminated in October, a month earlier than the latter (Fig. 1 and Table I). It was extremely rapid in May and especially in June,

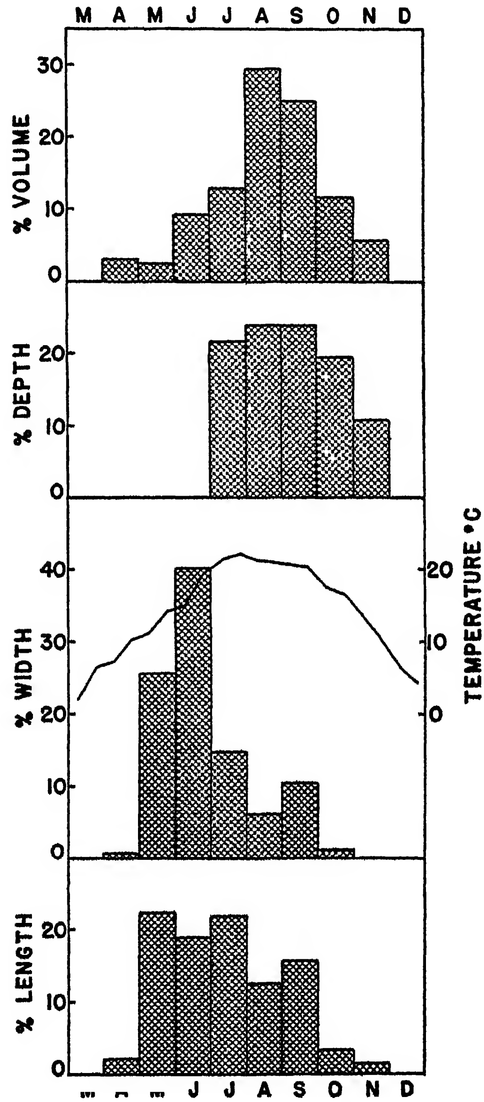

FIGURE 1. Per cent of increase in length, width, depth and volume of oysters during each month of the growing period. The total increase of each variate for the entire growing period, 1946 is taken as 100 per cent. Temperature curve is based upon semi-weekly records made at high water stages.

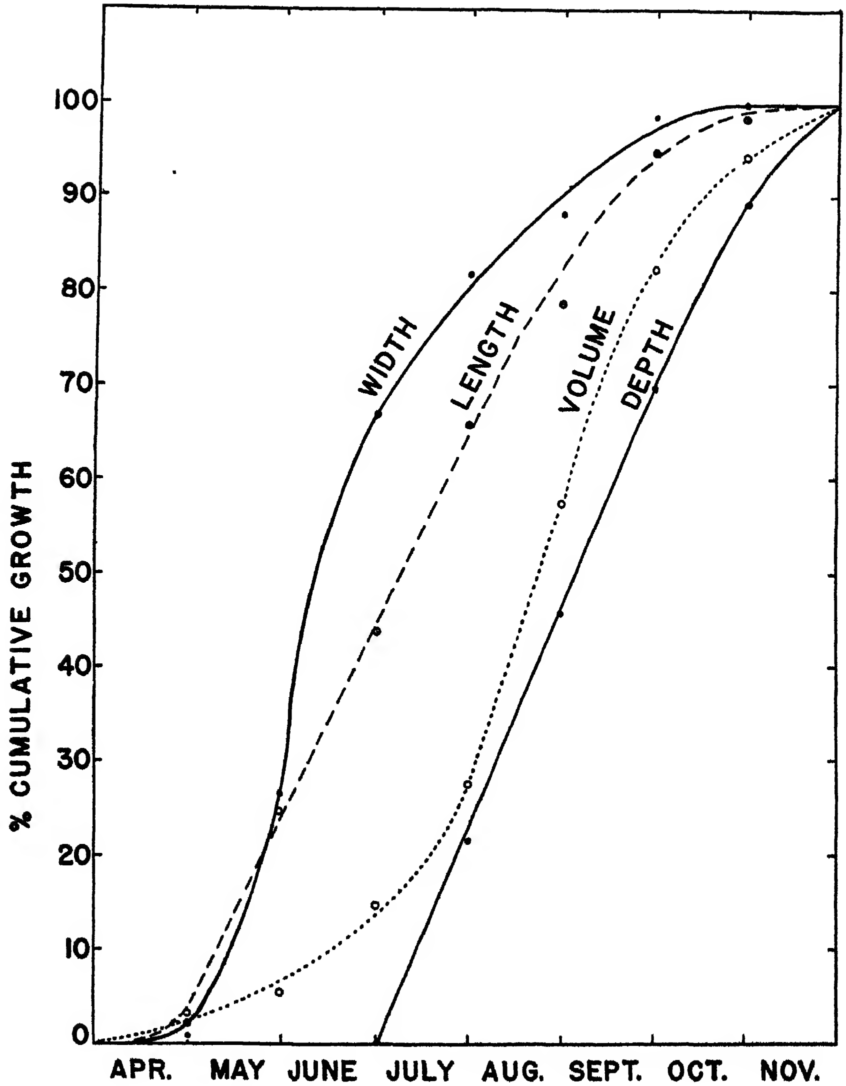

FIGURE 2. Per cent of cumulative growth in length, width, depth and volume of oysters recorded at the end of each month. The growth of each variate for the entire growing period of 1946 is taken as 100 per cent.

the latter month giving about 40 per cent of the total annual increase. In July, however, a sharp decrease was recorded. The decrease was even more pronounced during August.

Although the oysters increased in length, width, and volume during April, May, and June, the increase in the greatest depth was not appreciable until July (Figs. 1 and 2). During the first three months of the growing period the thinner parts of

the shells became thicker, but this change was not reflected in the greatest depth, or thickness, of the oysters. Nevertheless, the observations on the increase in the greatest depth are of interest because they indicate, as do the studies of the increase in volume, that an increase in depth is largely achieved during the second part of the growing period.

The increase in volume of the oysters, the same as the increase in length, continued from April through November (Figs. 1, 2 and Table 1). The greatest monthly increases were recorded during August and September, these two months giving approximately 55 per cent of the total annual increase in volume. As could be expected, the period of rapid increase in volume corresponded to that of greatest depth.

The marked increase in length and width of the oysters during May and June did not materially contribute to the increase in volume. This, of course, should be expected because newly formed shell-margins are very thin, displacing only small quantities of water.

Because the experimental oysters were individually numbered, it was possible to follow the increase in size of each individual from month to month throughout the entire growing period. This was done for two variates—length and volume. As usual, when working with a large number of animals, considerable individual differences were found. Nevertheless, the individual records showed the following interesting facts:

The maximum period during which oysters may grow in Milford Harbor is approximately of eight months' duration, extending from April to November, both months inclusive. However, only a small minority, comprising approximately 3 to 4 per cent of the entire group, grew during all the eight months, while for the majority of oysters the increase in length and volume was recorded only for five, six or seven months of the possible eight. About 3 per cent grew only three months and 10 per cent showed an increase in size for only four months, which were not always consecutive.

The chief increase in length and width of the oysters occurred during the first half of the eight-months' growing period, while the increase in the greatest depth and volume took place during the second half (Fig. 1). In this respect our observations are in agreement with those of Nelson (1922).

Not all the oysters began to grow in length and volume during the first month after the end of the hibernation period. Only about 48 per cent of the entire group increased in length in April, 49 per cent in May, while 3 per cent did not start growing until June. In volume, about 29 per cent began to show an increase in April, 40 per cent in May, 27 per cent in June, while 4 per cent did not show any increase until July.

Although it is true that we are usually concerned with the average animal, nevertheless, observations and records of unusually fast or slow-growing individuals are of significant biological value and interest because they may indicate that, within what may appear to be a homogeneous population, there may be distinct fast or slow-growing races. Some of the observations on individual oysters are given in the following paragraphs.

The greatest individual increment in length for the entire season was shown by oyster no. 22 which grew from 91.8 to 129.0 mm. in seven months, an increase of

37.2 mm. The smallest increase was shown by oyster no. 98 which grew from 77.6 to 85.3 mm., an increase of only 7.7 mm. This oyster grew in length only during two months out of a possible eight.

The greatest increase in volume for any individual was made by oyster no. 5, which increased from 104.0 to 176.0 cc., a total of 72 cc. in seven months. Oyster no. 17 showed an increase of only 8 cc., growing from 57.0 to 65.0 cc. in five months.

Individual records also made it possible to ascertain the maximum increase in length or volume of the fastest growing oysters for every month of the season (Table II). The largest monthly increase in length was made by oyster no. 90, which during July increased 15.2 mm., representing an increase of 17.9 per cent over the total length recorded at the end of June. During November the fastest growing oyster increased its length only by 3.4 per cent.

TABLE II

Greatest monthly increases in length or in volume shown by individual oysters. April–November, 1946. Milford Harbor

	April	May	June	July	Aug.	Sept.	Oct.	Nov.
Length								
Oyster no.	81	104	78	90	82	21	22	26
Old size in mm.	78.0	90.3	94.3	84.7	110.9	119.1	121.5	114.6
New size in mm.	82.5	100.8	105.6	99.9	117.9	129.1	129.0	118.5
Increase in mm.	4.5	10.5	11.3	15.2	7.0	10.0	7.5	3.9
% Increase	5.8	11.6	12.0	17.9	6.3	8.4	6.2	3.4
Volume								
Oyster no.	7	1	59	78	34	22	49	9
Old volume in cc.	73	78	73	72	79	98	106	128
New volume in cc.	78	82	84	87	99	127	122	136
Increase in cc.	5	4	11	15	20	29	16	8
% Increase	6.8	5.1	15.1	20.8	25.3	29.6	15.1	6.3

The greatest monthly increment in volume was made in September by oyster no. 22, which increased 29 cc. or 29.6 per cent over the volume recorded at the end of the preceding month.

It is significant that the greatest individual increases in length occurred during May, June, and July, that is, during the months when the group as a whole grew in length most rapidly. For the volume, both the greatest individual and group increments were noted in August and September (Fig. 1, Table II).

The records also show that the per cent of oysters increasing in length or volume varied considerably during different months. The increase was most common during July and August when almost all the oysters showed it, and the least noticeable in April and November.

Observations made on monthly growth of oysters in Milford Harbor suggest the following conclusions and deductions: Growth of the oysters, taken as a group, continued throughout the period extending from April to November, both months in-

cluded, without definite interruption during the spawning season. This observation is in agreement with the conclusions of several investigators working with other lamellibranch mollusks. For example, Belding (1912) found that the hard-shell clam, *Venus mercenaria,* grows very fast during July and August when its spawning is in progress. During these two months the clams show approximately 45 per cent of the total annual increase in the length of the shell. Belding (1931) also found that the soft clam, *Mya arenaria,* which in Massachusetts waters spawns during June, July, and August, shows during these three months approximately 55 per cent of the annual increase in the length. Coe (1945, 1947) observed that the California bay-mussel, *Mytilus edulis diegensis,* also grows during the spawning period.

In other lamellibranchs, however, the rate of growth may be appreciably diminished during the spawning season. Belding (1910) noticed such a decrease in the bay scallop, *Pecten irradians.* Coe (1947) thinks that the decrease in monthly increments in length of the Pismo clam, *Tivela stultorum,* in August is due "to the requirements of the reproductive system and the successive acts of spawning."

In the case of oysters, Nelson (1922) found that *O. virginica* of the New Jersey coast grows rapidly until the spawning period but more slowly thereafter, while Orton (1935) observed two main periods of shell growth of *Ostrea edulis,* one in spring and one in autumn.

A very rapid increase in the mean length and width of the oysters of Milford Harbor occurred during May and June, i.e., during the period of most active gametogenesis for the oysters of this region (Loosanoff, 1942). Apparently, the process of development and accumulation of gametes did not interfere with the growth of the shell, at least as far as the increase in length and width was concerned. This conclusion is well supported by observations on oysters which are conditioned every winter in our laboratory to develop ripe eggs and spermatozoa (Loosanoff, 1945). The oysters are brought from the beds in the hibernating state and after being kept at room temperature for several hours are placed in trays with running warm water. In a month or less, depending upon the temperature of the conditioning trays, the oysters are ripe. Yet, during this period of extremely active gametogenesis the majority of the oysters grow rapidly in length and width, forming new shell-margins which quite often are over 1.0 cm. This proves, of course, that gonad development and rapid growth of shell may proceed simultaneously.

Mass spawning of the experimental oysters was observed during the last few days of June. There is no doubt that these oysters continued spawning during July and that many of them completed spawning during that month. The latter point was ascertained by opening Milford Harbor oysters not used in the experiment. Therefore, we concluded that since July was the month of most active spawning and since the increase in length during that month was very rapid, it is apparent that the spawning activities did not sharply affect the rate of increase in the shell length. In this respect our conclusions differ from those of Orton (1928) who reported that the rate of growth of the European oyster, *O. edulis,* is considerably slowed down during the breeding period.

It may be tempting to explain the slowing of the growth in width of our oysters during July by ascribing it to the spawning activities. Such an explanation, however, does not appear to be very conclusive because three other variates showed an increase during that month (Fig. 1). Even if the rate of increase in width during

July was considerably slower than that observed in June, it still was comparatively rapid, occupying the third position among the eight months of the growing season. Moreover, our laboratory experiments on the conditioning of oysters for spawning in the winter gave us additional proof that spawning does not stop, or seriously decrease, the rate of shell growth. For example, in February, 1949, a group of 105 oysters was brought from Long Island Sound, where the water temperature was below 5.0° C., and after being measured was placed in warm running sea water at 25.0° C. At the end of the ninth day at this temperature the oysters spawned. Some of the spawning oysters had already at that time a new shell growth which measured over 1.0 cm. After spawning, the oysters continued to form a new shell for some time.

The slowing down of the rate of growth in length and width during August also should not be attributed to the spawning activities because of the considerations presented above. Furthermore, spawning was almost completed during July. Perhaps the slow growth could be more logically associated with the post-spawning stage, during which emaciated oysters are, presumably, not in condition to divert much of their energy into building new shell substance. This assumption is again easily invalidated because of the pronounced acceleration in the increase in volume noticed in August and in early September (Fig. 1).

There are some indications of possible physiological antagonism between the growth of oysters and the process of accumulation of glycogen in their tissues, a phenomenon commonly known as "fattening of oysters." In our waters, chief accumulation of glycogen in the meats of oysters occurs between the completion of spawning and until hibernation, thus covering a period of approximately three months, namely, September, October, and November. During this period the rate of increase in size and volume of oysters progressively diminishes (Figs. 1 and 2). Whether this decrease is due to the true antagonism of the different physiological functions, or merely reflects the changes occurring in the surrounding water, remains at present undetermined.

The changes of the water temperature and the monthly rates of growth of oysters showed only a partial relationship. It is true that the increase in length and width of the shells recorded in April, May and June was accompanied by a steady rise in temperature (Fig. 1). In July, however, the rate of increase in the width markedly decreased, although the temperature remained above 20.0° C., but such a presumably favorable condition was not reflected in the rate of increase in length and width. The comparatively slow rate of growth in length and width observed during October cannot be explained by the unfavorably low temperature, because during that month the average temperature was not lower than that recorded for May and the early part of June when the shells grew so rapidly.

A much clearer relationship was found between the monthly increments in volume and the changes in water temperature (Fig. 1). In spring and early summer the monthly increments increased simultaneously with the increase in temperature. The period of the most rapid monthly increases in volume roughly corresponded to the period of maximal seasonal temperature, while in the fall both showed a gradual decline.

In connection with these studies it was thought desirable to determine by experimental means the rate of growth of groups of oysters kept at different temperatures. This was done in the winter time because it was easier then to maintain in the lab-

oratory the desired temperatures merely by mixing definite quantities of cold and warm running sea water.

The warm sea water system, which is operated in our laboratory during the cold season, is regulated by a series of thermostats which control the temperature of the outflowing water. The temperature of our cold water is also very uniform. Therefore, in the winter time water of any temperature within the range of about 5.0° to 35.0° C. can be had by using constant level jars of cold and warm water and by regulating by stopcocks the flow from these jars into a mixing chamber until the desired temperature is obtained. From the mixing chamber the water is flowed into the trays or aquaria containing the oysters.

In the middle of February a shipment of four-year-old oysters, consisting of individuals of approximately the same size, was brought from the beds of Long Island Sound and placed for several hours in sea water of about 8.0° to 9.0° C. to let the oysters come out of hibernation. Then they were divided at random into four groups each containing 105 animals. After determining the average length and width of each group (Table III), the oysters were placed in trays with running water the temperature of which was brought up and then steadily maintained at approximately 10.0°, 15.0°, 20.0° or 25.0° C. All the trays were receiving the same quantity of water.

TABLE III

Average increase in length and width of oysters kept at temperatures of 10.0, 15.0, 20.0 or 25.0° C. from February 15 to March 16, 1949

Temperatures	10.0° C.		15.0° C.		20.0° C.		25.0° C.	
	L.	W.	L.	W.	L.	W.	L.	W.
Original measurements 2/15/49	92.1	70.3	91.5	68.6	92.5	70.1	94.0	70.1
Final measurements 3/16/49	93.4	71.3	99.9	76.0	100.1	77.3	98.5	73.2
Increase in mm.	1.3	1.0	8.4	7.4	7.6	7.2	4.5	3.1
% Increase	1.4	1.4	9.2	10.8	8.2	10.3	4.8	4.4

A month later the oysters were again measured (Table III). The 15.0° C. group grew best, showing at the end of the experiment an increase of 9.2 per cent in length and 10.8 per cent in width of shell. The maximum increase in length shown by the fastest growing oyster of this group was 21.0 mm. The growth of oysters kept at 20.0° C. was almost as fast as that of the 15.0° C. group. However, the 25.0° C. group grew much more slowly than the two above mentioned, and the 10.0° C. group showed only a slight increase in size. The maximum increase in length attained by the fastest growing oysters of the 25.0° and 10.0° C. groups was 14.0 and 5.0 mm. respectively.

In the 15.0° C. group all the oysters showed new growth and in 20.0° C. only one individual did not form new shell. In the 10.0° and 25.0° C. groups, however, many oysters did not grow.

Examination of the new shell growth showed that its character was different in the different groups. In the lowest group the new shell was, at the end of the experiment, still transparent, soft and flexible. In the 15.0° C. group, however, the

new shell substance was already becoming harder and more brittle, and only the most recently formed part, confined to the edges of the shell, was still soft and flexible. This condition was even more pronounced at higher temperatures but, nevertheless, even in those groups many oysters were still forming new growth during the last days of the experiment.

Thus, under the conditions under which the experiment was run, the oysters grew most rapidly at temperatures of 15.0° and 20.0° C. Therefore, the optimum temperature range for their growth was either confined between these two temperatures or, what is more probable, extended a degree or two outside these two limits giving a range from approximately 13.0° to 22.0° C. It is interesting that the rapid increase in length and width shown in the spring and early summer by the oysters grown in Milford Harbor took place during May and June, in other words, when the water temperature was within the range given above (Fig. 1).

Our laboratory observations on the growth of oysters at different temperatures were, however, of too short a duration to find whether, if the experiment had been continued for several more months, the growth of each group would have proceeded at its original rate or would have shown some important changes. For example, it is possible that if the experiment had been prolonged, the rate of growth of the fast growing groups of 15.0° and 20.0° C. would have gradually decreased and, perhaps, eventually stopped, while the growth of the 10 0° C. group would have proceeded at the same or even at a somewhat faster rate than that shown during the first month of observation. It is planned to find the answer to this question in the near future.

With our present knowledge, it is impossible to estimate accurately the effect of food upon the growth of oysters. In a basin, such as Milford Harbor, where the tidal currents are swift and where the difference between high and low water levels may be as much as 9 feet, the quantity and quality of the material suspended in the water flowing over the oysters changes continuously. Even if it were possible to collect samples continuously, such samples would be of only limited value because many forms composing nanno and ultraplankton disintegrate almost immediately after collection. Thus, even if the quantity of material suspended in the water could be somehow determined, the quality of part of it would remain unknown.

Perhaps the greatest handicap facing the students of the role of food upon growth and other phases of the physiology of oysters is our lack of definite knowledge as to what really is the food of these mollusks. A full discussion on this subject is not the purpose of this article—those interested are referred to a summary published recently (Loosanoff and Engle, 1947). Briefly, however, while one school of investigators assumes that living plankton is the main ingredient of the oyster diet, the second school led by Coe (1945, 1947) is of the opinion that most of the nutrition of oysters, clams, mussels and other filter-feeding bivalve mollusks is derived from the intra-cellular digestion of particles of detritus originating from the disintegrated cells of marine animals and plants. Coe's conclusions appear to be well supported but, nevertheless, the issue is still debatable and not finally solved. As long as it remains in this stage, and as long as the value of different components of plankton and detritus are undetermined, it will remain impossible to formulate intelligently the relationships between the quantities or qualities of food present in the water over the oyster beds and the various aspects of the physiology of oysters or other mollusks closely related to them.

The difficulties of solving these problems are further complicated because oysters, and probably some other lamellibranchs, can feed efficiently only when the concentration of plankton (Loosanoff and Engle, 1947) or turbidity-creating substances as detritus or silt (Loosanoff and Tommers, 1948) do not exceed certain thresholds. If such thresholds are exceeded, the normal existence of mollusks becomes impossible. Thus, in addition to determining what organisms or materials constitute oyster food, it will also be necessary to determine their optimum concentrations in relation to the feeding and growth of oysters.

It should also be mentioned that a rapid increase in length and width of shell does not necessarily indicate that the oysters are growing under favorable conditions. For example, on several occasions at Milford Laboratory the oysters discarded after being used in the experiments were crowded in small aquaria through which only a trickle of water passed. Yet, within a short time some of them showed new shell growth. This growth was formed despite the fact that the oysters were not receiving enough food and that the water in which they were kept contained large quantities of waste products. Similar observations were made on oysters kept in heavy concentrations of micro-organisms, such as Chlorella and Nitzschia, which interfered with the normal feeding. The oysters eventually died (Loosanoff and Engle, 1947) but, nevertheless, even if their meats were emaciated, new shell growth was forming shortly before their death. These observations suggest that the factors involved in the growth of oysters are rather complex and at present not well understood.

The data and the conclusions on the monthly increase of oysters offered in this article are based upon only one year of observations. It is possible that during some years, when conditions are unusually favorable and the water temperature is considerably above normal during March or December, a slight increase in the size of the shells may be noted during these months. It is also possible that the monthly increases of the variates in different years would differ somewhat from those shown in our Figure 1. Nevertheless, it is believed that such variations would not basically change the trend of growth during the year.

Summary

1. The oysters grown in Milford Harbor did not increase in size, volume or weight during the hibernation period. However, if by some artificial means the temperature of the water is kept above the hibernation point, the oysters will continue to grow in the laboratory even in the middle of winter.

2. The maximum period during which oysters may grow in Milford Harbor is approximately of eight months' duration extending from April to November, both months inclusive. Only a small minority comprising approximately 3 to 4 per cent grew during all the eight months, while the majority grew only for five, six, or seven months. Some oysters did not start growing in length until June, and in volume until July.

3. The increase in length was most rapid during May, June, and July, representing 22.57, 19.03 and 22.12 per cent respectively of the total annual increment. The growth in width was especially rapid in June, giving 40 per cent of the total annual increase. The increase in the greatest depth was not appreciable until July.

4. The increase in volume continued from April through November; the greatest

monthly increases were recorded during August and September; these two months combined gave approximately 55 per cent of the annual increase in volume.

5. The increase in size was most common during July and August, when almost all the oysters showed it, and least noticeable in April and November.

6. The process of gametogenesis did not interfere with the growth of the shell, at least as far as the increase in length and width was concerned.

7. The spawning activities did not adversely affect the rate of increase in length and in volume.

8. The chief increase in length and width of the oysters occurred during the first half of the growing period, while the increase in depth and volume was most pronounced during the second half.

9. Changes in the rate of growth in length and width showed only partial relationship with changes in the water temperature. However, a rather definite relationship was found between the changes in the rate of increase in volume and changes in the water temperature.

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THE PREZONE PHENOMENON IN SPERM AGGLUTINATION

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The agglutination of the sperm of certain marine invertebrates by a substance known as fertilizin from the jelly hull of the eggs of the same species resembles serological reactions. This was first pointed out by Lillie (1919), and the concept has been expanded more recently by Tyler (cf. Tyler, 1948) on the basis of various features of the interactions of fertilizin and antifertilizin. One of these features is the occurrence of the zone phenomenon.

The zone phenomenon is typical of ordinary serological reactions (see Marrack, 1938). It is typified by the occurrence of maximum amounts of agglutination or precipitation when antigen and antibody are mixed in certain proportions. Increasing or decreasing the amount of antigen, or (in some cases) of antibody, above or below the optimum value results in decreasing amounts of agglutination or precipitation. The zones are also revealed by the occurrence of more rapid reactions (agglutination or precipitation) when the reagents are mixed in the proportions of the optimum zone than when mixed in other proportions. The mutual multivalence theory of antigen-antibody reactions proposed by Marrack (1938) and by Heidelberger (1939) offers an interpretation of the occurrence of zones. According to this theory, antigen and antibody molecules are both multivalent with respect to the mutually complementary groups by which they combine, with two or more such groups on each molecule. This would permit the formation of large aggregates of antigen and antibody. If the antigen were a cell surface, as in agglutination reactions, the process would result in agglutination. This hypothesis has received substantial support from the recent work of Pauling et al. (1944). Tyler (1940b) suggested that this line of reasoning could be applied to the agglutination of sperm by homologous fertilizin.

The occurrence of a prezone in an agglutination reaction may be interpreted in at least two ways according to the "framework" theory of antigen-antibody reaction. The prezone in agglutination reactions occurs in the region of highest agglutinin concentration; thus there would be such an excess of agglutinin molecules that the combining groups on the surface of the cell would each bind a separate agglutinin molecule, and no single agglutinin molecule would thus be likely to combine with more than one cell. This would result in little or no agglutination in very high concentrations of agglutinin and in increasing amounts of agglutination as the agglutinin concentration is lowered.

Another interpretation would be that "univalent agglutinin" molecules (agglutinin molecules with only one specific combining group each) are present along with the multivalent agglutinin molecules. Thus in regions of high agglutinin concen-

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tration, the univalent agglutinin would be able to combine with many of the specific combining groups on the agglutinogen surface and thus inhibit agglutination by the multivalent agglutinin. In any particular case, one or the other or both interpretations may apply.

Tyler (1940a) noted the occurrence of the zone phenomenon, as exhibited primarily by rates of agglutination, when sperm of the limpet Megathura were mixed in various proportions with homologous egg water. With a given amount of sperm, for example, the most rapid agglutination does not occur with the strongest egg water but with lower concentrations. Further decrease in egg water concentration, of course, gives slower and less extensive reactions until the end point of no visible reaction is reached.

It was of interest to investigate the possible occurrence of the zone phenomenon in another group of animals, the sea urchins, which differs from the molluscs in certain details of the agglutination reaction of sperm with egg water (see Tyler, 1940a, 1941). Also it seemed desirable to examine this on the basis of degree of agglutination rather than simply on the basis of rate of reaction.

Materials and Methods

Sperm and fertilizin of the sea urchin *Lytechinus pictus* were used for the experimental work. The testes were carefully removed from the animals to avoid contamination with body fluid, and the "dry" sperm was allowed to extrude. This was filtered through bolting silk and stored in small sealed flasks at 8° C. until used. For testing purposes, a 1 per cent suspension of the sperm in filtered sea water was prepared. The fertilizin solution was prepared by allowing a thick suspension of eggs to stand for several hours, centrifuging it and using the supernatant, or by extraction with acid sea water as described by Tyler (1940b). No consistent differences were noted in the action of the fertilizin prepared by these two methods. For observing the agglutinating activity of the fertilizin preparations, serial dilutions in filtered sea water with a final volume of 1.0 ml. were prepared in 10×75 mm. test tubes, and 0.1 ml. of 1 per cent sperm suspension added. The tubes were agitated to mix the contents, and the degree of agglutination read macroscopically at 15 minutes after adding the sperm. The degree of agglutination was expressed in the customary 0 to 4 + terminology.

Experimental

In general, when constant amounts of homologous sperm are added to serial dilutions of fertilizin, the degree of agglutination observed is proportional to the fertilizin concentration. It should be pointed out that Lytechinus sperm does not show the rapid reversal of agglutination characteristic of the *Strongylocentrotus purpuratus* sperm plus fertilizin system. In the case of Lytechinus, the agglutination may persist for several hours, although this varies with sperm from different individuals. Extremely fresh sperm often shows a very low degree of agglutination when fertilizin is added, but after aging for several hours in the refrigerator, the degree of agglutination usually increases.

With some samples of Lytechinus fertilizin, it was noted that there was little or no agglutination in the highest fertilizin concentrations, although good agglutination

occurred with lower concentrations. This resembles the prezone observed occasionally in a bacteria plus specific antiserum system, such as that described by Coca and Kelley (1921) for certain antisera against *Klebsiella capsulata*.

Wiener (1944) suggested that the prezone in the agglutination of Rh positive erythrocytes by certain anti-Rh sera was due to the presence of what he called "blocking antibodies," i.e., anti-Rh antibodies capable of specifically combining with Rh positive cells but unable to agglutinate them. The fact that the antibody had actually combined with the cells was established by showing that these cells could not be subsequently agglutinated by normal anti-Rh antisera.

It has been shown that treatment of fertilizin with heat, x-rays, and ultraviolet light (Tyler, 1941; Metz, 1942) converts it into the "univalent" form, that is, a form where it can no longer agglutinate the sperm, although it is still capable of combining specifically as may be shown by inhibition tests. This probably is due to the splitting of the fertilizin molecule into fragments containing only one effective group each.

In the first experiments conducted in the present work, the fertilizin was irradiated with ultraviolet light (source described by Spikes, 1944) to produce univalents. Later it was found that irradiating the fertilizin with visible light in the presence of a photo-sensitizing dye (eosin) according to the first method described by Tyler (1945) gave better results.

TABLE I

Degree of agglutination in the dilution of irradiated fertilizin indicated

Hours of irradiation	Titer of fertilizin 0	1	3	9	27	81	243	729	2187	6561
0	0	+++	+++	++++	++++	++++	+++	+	±	0
4	0	+	++	+++	+++	+++	++	+	±	0
20	0	0	+	++	+++	+++	++	±	±	0
26	0	0	0	+±	++	+++	++±	+	±	0
40	0	0	0	0	+	++±	++±	+	±	0
65	0	0	0	0	0	++±	+++	0	0	0

It was found that by using preparations containing univalent fertilizin along with the agglutinating type, the width of the prezone could be increased to a remarkable degree without appreciably affecting the beginning of the postzone. This is shown in Table I, which indicates the degree of agglutination resulting when constant amounts of sperm were added to serial dilutions of fertilizin irradiated with 750 foot candles of daylight-type fluorescent light in the presence of 0.2 per cent eosin for the number of hours indicated. If fertilizin which had been irradiated until it no longer produced any agglutination was added to normal fertilizin, a prezone was also produced.

Explanations for this induced prezone in terms of the "framework" theory of agglutination may now be suggested. If part of the molecules in a sample of fertilizin are converted into the univalent form by the irradiation, serial dilutions of this made, and constant amounts of sperm added to each dilution, there may be sufficient univalent fertilizin in the region of high fertilizin concentration to combine

with all of the combining groups of all of the sperm present, and thus prevent agglutination. In setting up the serial dilutions the fertilizin solution is successively diluted, while the amount of sperm added to each dilution remains constant. Therefore, a dilution would be reached where there would no longer be sufficient univalent fertilizin to combine with all of the sperm, thus leaving some over to be agglutinated by the normal (multivalent) fertilizin present.

An alternative suggestion would be that the irradiation breaks the fertilizin molecule into a number of fragments which are still multivalent. Then when the sperm were added there would be such great competition for the combining groups on the sperm surface that it would be difficult for one fertilizin fragment to combine with more than one sperm. This again would result in a lower degree of agglutination in the region of high fertilizin concentration. There is at least one serious objection to this latter explanation, however. If the number of multivalent fragments was increased by the irradiating process, it would be expected that the endpoint of the agglutination reaction would occur at a higher dilution of the fertilizin than before the irradiation. An examination of the data in Table I, however, shows that the endpoint was moved in, rather than outward, to a region of higher fertilizin concentration.

From either of the above interpretations it could be predicted that multivalent fertilizin should be present in the tubes in the prezone region. This was shown to be true by successive absorptions with small quantities of sperm. The first few quantities of sperm added to the prezone dilutions were not agglutinated, but a point was soon reached where added sperm was strongly agglutinated. Presumably at this point (according to the first explanation suggested above) all of the univalent fertilizin was absorbed out so that the multivalent fertilizin present was able to combine with and agglutinate the sperm. The results of a typical experiment of this type are shown in Table II.

TABLE II

Degree of sperm agglutination in various dilutions of 65 hour irradiated fertilizin after successive absorptions with sperm

Number of times absorbed with 0.2 ml. of 2% sperm	Titer of fertilizin 0	3	9	27	81	243	729
1	0	0	0	0	++±	+++	+
2	0	0	0	++	+++	±	0
3	0	0	±	++++	+	0	0
4	0	+	++	++	±	0	0
5	0	+++	+++	±	0	0	0
6	0	+++	++++	0	0	0	0
7	0	+++	++	0	0	0	0
8	0	++++	0	0	0	0	0
9	0	++++	0	0	0	0	0
10	0	++++	0	0	0	0	0
11	0	++++	0	0	0	0	0
12	0	++++	0	0	0	0	0
13	0	++	0	0	0	0	0
14	0	±	0	0	0	0	0
15	0	0	0	0	0	0	0

The work reported above is regarded as further evidence of the similarity of the reactions between the specifically combining substances of sperm and eggs to serological reactions.

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AN ELECTRON MICROSCOPE STUDY OF THE EGG MEMBRANES OF MELANOPLUS DIFFERENTIALIS (THOMAS)[1,2]

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Extensive research has been carried out on the origin and the physical and chemical properties of the membranes surrounding the egg of the grasshopper *Melanoplus differentialis* (Thomas). The results of Campbell (1929), Jahn (1935a, 1935b, 1936), and Cole and Jahn (1937) seem most helpful in understanding its physical and chemical nature, while Slifer (1932, 1937, 1938a, 1938b), and Slifer and King (1934) give a clear picture of the structural relations of the egg membranes during embryonic development. It is the purpose of this paper to present results obtained from a study of the egg membranes with the aid of the RCA Electron Microscope, Model EMU-2B.

The outer membrane of the grasshopper's egg, the chorion (about 20 μ thick), is secreted by the cells of the maternal ovariole epithelium which enlarge during yolk deposition. Investigators seem to disagree regarding the formation and continuity of the vitelline membrane which lies just inside the inner surface of the chorion (Slifer, 1937). Since this membrane appears to become fragmentary as soon as embryonic development begins, it was not studied with the electron microscope.

At the time of laying, the egg, which has broken away from the ovariole epithelium, passes down the oviduct and out of the ovipositor into a pod made up of from 10 to 150 eggs. During the development of the blastoderm and its differentiation into germ band and serosa, very little change occurs in the egg membranes. The serosa cells migrate peripherally and completely surround the yolk and germ band by the fifth day (at 25° C.). They appear just inside the chorion as large, flat cells, with dense elliptical nuclei. During the sixth day, the serosa cells secrete on their periphery a non-chitinous (Campbell, 1929) membrane called the yellow cuticle (Jahn, 1935a, 1935b, and 1936). It is usually complete by the beginning of the seventh day at 25° C. Jahn (1936) found this thin membrane ($< 1 \mu$) to show a high degree of ionic impermeability, and it may be closely related chemically to the cuticulin of *Rhodnius prolixus* (Wigglesworth, 1933).

The serosa cells also secrete a white fibrous membrane differing structurally and chemically from the yellow cuticle, and lying just inside of it. This layer, which gave Campbell and Jahn a positive chitosan test, is the white cuticle. Slifer's (1937) microscopical examination showed it "to be composed of innumerable fine threads tangled closely together." The deposition of this layered membrane (about 20 μ thick) requires one week at 25° C. (Slifer, 1937). She concluded, "The yel-

[1] This study was made possible by the cooperation of Dr. Titus C. Evans, Director of Radiation Laboratories, and technical assistance of Dr. Clinton D. Janney.

[2] Aided by grant from the National Health Institute, administered by Professor J. H. Bodine.

low layer confers a high degree of impermeability; while the white layer is responsible for a greatly increased toughness and resistance to mechanical injury."

As the embryo develops, the chorion, if allowed to dry, cracks into an irregular pattern. The yellow cuticle is broken during the hatching process, but the embryo apparently is not strong enough to break the tough fibers of the white cuticle. Just before hatching, the latter, or the major portion of it, is digested away, making possible the emergence of the nymph. Slifer (1937, 1938b) has submitted evidence that the enzyme which is responsible for this digestion is produced by the pleuropodia.

The investigation of these membranes with the electron microscope requires special techniques and much patience. Since the electron beam is capable of penetrating tissue only about 1 μ in thickness, clear photographs of structures can only be made if the thickness is kept below 0.5 μ. There are many ways proposed for the preparing of extremely thin sections. All methods thus far noted in the literature fall into two categories: *A*. High speed microtomes, or *B*. Variations in the mechanics of sectioning.

Among the high speed microtomes which have been used and recommended are the "Cyclone Microtome" of O'Brien and McKinley (1943) and the two models by Fullam and Gessler (1946). One of the most difficult problems of the high speed microtome seems to be the locating of the sections after they are cut.

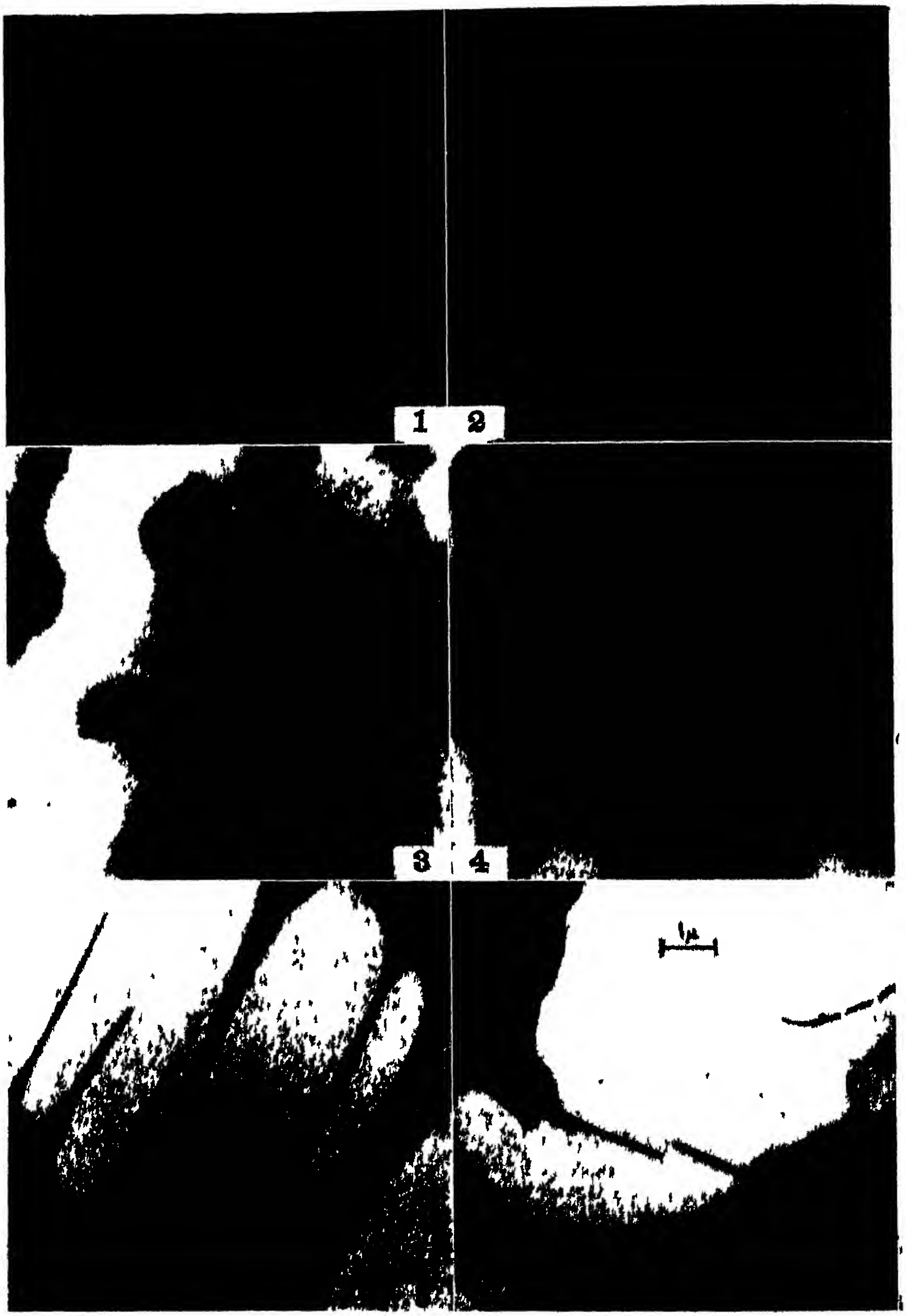
The earliest workers with the electron microscope tried many variations of geometry, mechanics, and chance. One of the methods, which involves a great deal of chance, is to cut the thinnest sections possible on the conventional microtome. These sections are re-embedded and re-sectioned, the operator hoping to obtain at least one very thin section out of many original cuts. The most commonly tried method, and the method used in this study, was the cutting of wedge-shaped sections on the conventional microtome as described by von Ardenne (1939), and Richards, Anderson and Hance (1942). From these a great many fine electronmicrographs have been produced. A very successful method used by Pease and Baker (1948) is the modification of the conventional Spencer Model 820 rotary microtome by decreasing the pitch of the diagonal backing plate to enable the cutting of 0.1 μ thick sections from doubly embedded material.

Another method of preparing sections, which has been used by Richards and his associates at the University of Minnesota, is to choose material which is naturally very thin. The results of these studies are reported by Richards and Anderson (1942), Anderson and Richards (1942), Richards and Korda (1947), and Richards and Korda (1948). The present investigator has employed this method in securing electronmicrographs of the yellow cuticle.

All membranes used were obtained from the eggs of animals kept under laboratory conditions as described by Boell (1935). To obtain the extremely thin sections of the membranes the following methods were used:

A. Fresh yellow cuticle. As stated above, the yellow cuticle is deposited by the serosa cells about the sixth day after the eggs are laid. Six-day-old eggs were placed in sodium hypochlorite solution to dissolve off the chorion (Slifer, 1945). The only membrane left enclosing the egg at this age is the yellow cuticle. The yellow cuticle of the egg was ruptured in isotonic saline solution, and a piece of it (after rinsing in isotonic saline solution and distilled water) is placed on the object screen of the electron microscope.

Plate I



B. Preserved sections of chorion and white cuticle.
   1. Geometrical method. One end of eggs at different stages of development was cut off, and the eggs fixed in Bouin and embedded in paraffin (56° MP) by the usual method A number of eggs were embedded parallel in each paraffin block. The blocks were sectioned longitudinally or thereabouts in the conventional microtome at settings from 2–10 μ (Dempster, 1942). Some of the above eggs were prepared leaving yolk and embryo in the membranes, and some were prepared with the yolk and embryo removed before fixing. Little difference was noted in the results The only sections having wedge characteristics were those cut from the eggs at the beginning and ending of the sectioning. These sections were placed in xylol to dissolve out the paraffin before being mounted on the object screen of the electron microscope
   2 Modifying Spencer Model 820 Rotary Microtome. With this modification, eggs were doubly embedded as described by Pease and Baker (1948).

The placing of the sectioned material upon the object screen or grid near the center is not without problems. Since the grid wires are opaque, they always obscure a part of the material from view.

Since the limit of adjustment of the holder is only about five meshes of the grid in diameter, the exact centering of the specimen is critical. In sections carefully prepared and mounted, it may turn out that the material to be observed will have a location behind a wire of the grid. The super-drying of the specimen in the vacuum chamber, plus the "baking" it receives from the electron beams, renders the material so fragile and brittle that its relocation on the grid is next to impossible.

Results

An examination of the electronmicrographs with their titles and explanations reveals the structure of the membranes of the grasshopper egg. These figures are presented from the many pictures taken, as typical of the materials examined. In general, as the eggs become older, the chorion and yellow cuticle become electronically more dense. Thinner sections of older membranes were necessary before the material could be viewed or the electronmicrographs taken.

Figures 1 and 2 of the chorion (using wedge-shaped preserved sections) indicate that it appears to have no clearly resolvable internal structure. The shades of gray of the electronmicrographs vary greatly with the thickness of the sections. Figure 1 is a rather thick section of chorion, while Figure 2 shows a thinner section, and the additional thickness of torn yellow cuticle is at the edge.

Plate I

Figure 1 At edge of chorion from 14 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 2 Chorion from 14 days postdiapause eggs. Torn edge of white cuticle at top. Preserved specimen. × 13,000.

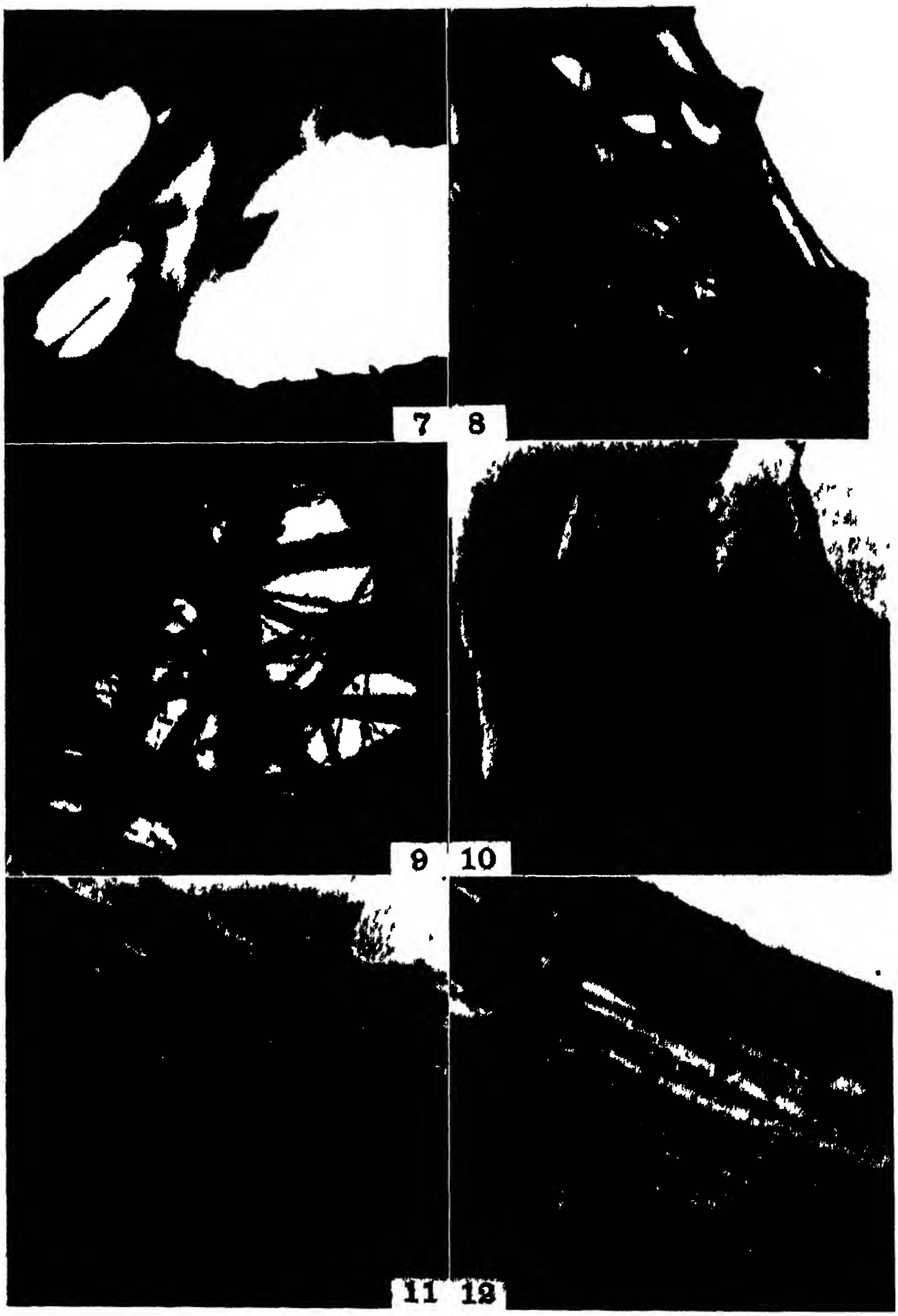
Figure 3. Yellow cuticle from 6-day-old eggs. Fresh specimen. × 13,000.

Figure 4. Same as Figure 3.

Figure 5. Stretched yellow cuticle from 6-day-old eggs. Fresh specimen. × 13,000.

Figure 6. Stretched yellow cuticle from 16 days postdiapause eggs. Preserved specimen. × 13,000.

Plate II



Since wedge-shaped sections were used, no exact measurements of thickness were possible. All estimates or comparisons of thickness were the result of noting the distance from the thin edge of the wedge. Cross sections of preserved chorion, when stained with Delafield's hematoxylin and eosin or with Mallory's triple stain, appear under the high power oil immersion lens of the light compound microscope to be composed internally of short uneven fibers (Slifer, 1937, 1938a). However, the unstained, longitudinal, wedge-shaped sections of the chorion examined in the electron microscope seem to reveal no indications of a fiber-like structure. (See Figs. 1 and 2.)

Figures 3 and 4 of the yellow cuticle give evidence of the "minute ridges and tubercles" as described by Slifer (1937). These appear on the outer surface of this membrane. The ridges give greater thickness to the yellow cuticle and are believed to be responsible for the "Dalmatian-dog" pattern, which appears to be larger in the fresh material (Figs. 3 and 4) than in the preserved specimens (Figs. 13 and 14). This difference in size may be due to variations in material and shrinkage of the preserved material.

Figure 5 shows the results of stretching the fresh yellow cuticle which occurs as it dries in the electron microscope. Even in these stretched strands, variations in thickness are apparent. Figures 5 and 6 show preserved yellow cuticle which has been pulled to the point of breaking. Figures 5 and 6 both seem to indicate that there is stretching before the strands break. Note the blunt ends of the broken strands.

Figures 8 through 12 show the fibrous layered structure of the white cuticle. It was discovered that if the wedge-shaped sections were stained in eosin before being mounted on the grid in the electron microscope, the fibrous structure was largely obliterated. Since this was interpreted as an artifact, all stains were omitted on membranes employed in this study.

Decided differences of structure between the yellow and white cuticles, as evidenced in this and previous studies, point to the serosa cells as embryonic in nature and differing biochemically during development. The ability of the serosa cells to secrete two different structures or membranes, the yellow and the white cuticle within the same egg, indicates a similarity of function to the epidermal cells of insects (Wigglesworth, 1948).

Figures 8 and 9 show an extreme variation in the size of the fibers. There are indications of individual fibers and bundles of fibers both being present in the same white cuticle. No explanation is offered for the nodules on the fibers which are

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Plate II

Figure 7. Stretched yellow cuticle appearing at crack in chorion from 16 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 8. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 9. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 10. Stretched white cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 22,200.

Figure 11. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

Figure 12. White cuticle fibers from 11 days postdiapause eggs. Preserved specimen. × 13,000.

PLATE III

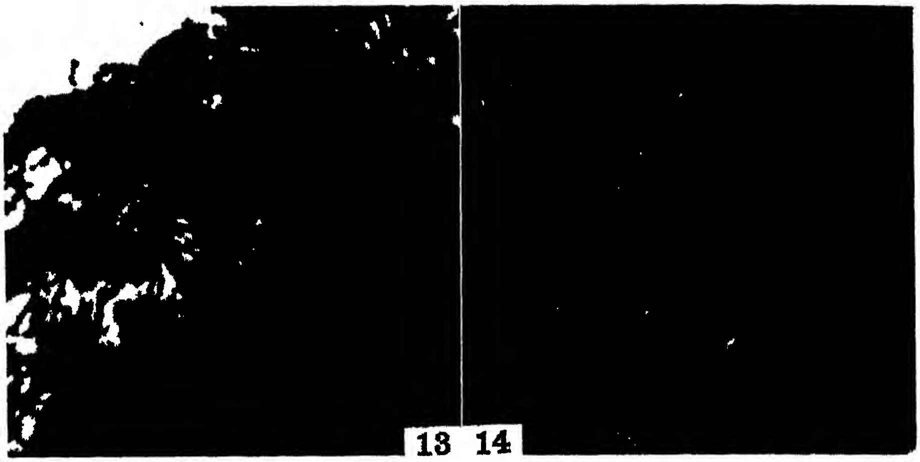

FIGURE 13. Yellow and white cuticle from 11 days postdiapause eggs. Preserved specimen. × 13,000.

FIGURE 14. Yellow and white cuticle from 11 days postdiapause eggs. Preserved specimen. × 13,000.

especially noticeable in Figure 9. Figure 10 shows the results of stretching white cuticle.

Figures 13 and 14 are combinations of yellow and white cuticle taken near the boundary of the two tissues. These electronmicrographs illustrate again the close proximity of the two cuticles, as parts of each may be viewed in one thin section. The "Dalmatian-dog" pattern is smaller in figures from preserved specimens than in Figures 3 and 4 from fresh yellow cuticle.

SUMMARY

Electronmicrographs of the grasshopper egg membranes by the methods used show that:

A. There is no clearly resolvable internal structure of the chorion.
B. The yellow cuticle has no clearly resolvable internal structure, but has varying thicknesses due to minute ridges or projections on its outer surface.
C. The white cuticle is fibrous in structure.

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PHOSPHATASES IN NORMAL AND REORGANIZING STENTORS

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The histochemical demonstration of phosphatase activity in *Stentor coeruleus*, although desirable in its own right, was undertaken primarily to determine to what extent such a study might amplify data obtained earlier (Weisz, 1949) with regard to metabolism and differentiation during normal and reorganizational stages in the life cycle of Stentor. Enzyme activity was studied in the normal, vegetative animal to provide a frame of reference, and this is compared with analogous data on starvation, regeneration, physiological reorganization, and vegetative division.

EXPERIMENTAL

In Stentor, phosphatases cannot be demonstrated with the usual "alkaline" techniques (e.g., Lillie, 1948). Air-dried (cf. below) or acetone-fixed test slides always yield precipitates in the same regions and in the same intensity as control slides prepared by omitting incubation or by substituting a calcium nitrate incubation for the treatment with substrate. Acid phosphatase activity, on the other hand, can be visualized.

The technique was adapted from Gomori's (1941) original method to demonstrate acid phosphatase activity. Stentors of appropriate stages were put on slides with a minimum of water, and after the organisms had been oriented, all excess water was drained off and the preparations were dried in a gentle air current from a fan. This method simultaneously flattens the animal to section thinness and fastens it to the glass. The preparations were then treated directly with the substrate.

In initial exploratory tests the usual method of fixation was tried by dropping living Stentors into chilled acetone before treating them with the substrate. It was found, however, that with this procedure much of the subsequent impregnation potential is lost, possibly due to partial enzyme inactivation by acetone (Stafford and Atkinson, 1948), possibly also due to a leaching out of some of the enzyme in the washing process (Barthelmez and Bensley, 1947). Air-drying on the other hand gave maximal and consistent results.

Of a number of substrates tried, sodium glycerophosphate was found to give the best results (cf. also Gomori, 1949), and this substrate, buffered to pH 4.7, and allowed to act for 15 to 20 hours at 25° C., was consequently used routinely. Post-incubation treatment followed the sulfide technique as outlined by Gomori. Control tests were carried out both by omitting incubation, and by poisoning the substrate with sodium fluoride (M/1000).

Recent work has raised some doubt whether the precipitates obtained by this method represent correct visualizations of phosphatase, and whether the loci of the precipitates correspond precisely to the *in vivo* sites of enzyme activity. Non-enzymatic impregnation of certain tissues by lead salts is known to occur (Lassek,

1947), especially during long incubation. The extent of this error can be estimated, however, by running controls in poisoned substrates. Inasmuch as in Stentor such control preparations do not reveal any precipitate, non-enzymatic impregnation probably does not occur to any appreciable extent under the present conditions of testing; the lead sulfide deposits obtained in the experimental material may thus be regarded presumptively as visualizations of enzyme activity. Nevertheless, in view of the possibility of enzyme shifting during the testing procedure (Barthelmez and Bensley, 1947), caution is warranted in interpreting the results, both with respect to the specificity and the localization of the reaction.

Examination of about 50 Stentors has shown that lead deposits are always found in a number of definite, circumscribed regions. In the ectoplasm, the deposits are centered in the basal granules of the body cilia and the membranelles. This gives the impression that the entire gullet and the peristome band are heavily impregnated, and that the longitudinal rows of body cilia are underscored with dark brown deposits. In the endoplasm, precipitates are particularly constant and abundant around the macronuclear nodes, but no deposits are observed within the nodes themselves. Heavy deposits are also found on the surface and probably within the endoplasmic vacuolar fat reserves (cf. Weisz, 1949), as well as in the immediate vicinity of the gastrioles. (In contrast, preparations fixed in acetone before incubation reveal only light deposits in the circumnuclear site, and no other part of the organism is impregnated.)

In starvation, a gradual decrease in the phosphatase reaction becomes manifest. Deposits in the fat vacuoles and near the gastrioles disappear first. By the time the oral area is about to be resorbed, only the regions around the macronuclei, and the basal granules of the membranelles, still yield a faint reaction (at this stage the preparations resemble those of normal animals which had been fixed in acetone). After the degeneration of the oral area even the circumnuclear activity soon disappears, and no part of the animal reveals any lead sulfide deposits. The data for physiological reorganization and vegetative division are rather parallel, and may be discussed together. The normal sites of activity largely persist unchanged throughout both types of reorganization. The point of interest centers around the regions in which new peristome bands are differentiated, anteriorly in physiological reorganization, and at mid-body in division (cf. Weisz, 1949). In every case, as in normal membranelles, newly differentiated membranelles show a high degree of activity in their basal granules. Such activity, however, can never be observed before the membranelles themselves have formed and are functional. In areas adjacent to newly formed peristome sections, i.e., in areas in which new membranelles will appear within a short time, activity is not yet evident.

Tests carried out on regenerating posterior fragments afford another opportunity to check on this point. Since the time at which new membranelles appear in a Stentor fragment is known (Weisz, 1948), it is possible to test for phosphatase before as well as after peristome new-formation. Such paired tests can be carried out on fragments obtained from the same animal. This was done, with results as above: presumptive sites of newly differentiating membranelles do not reveal any deposits; the latter become manifest only when the membranelles themselves can first be seen in an active state.

Apart from these differences in the presumptive oral area, regenerating frag-

ments do not differ from normal intact animals in the extent and the localization of the sulfide deposits.

If the deposits may indeed be regarded as visualizations of acid phosphatase activity, these observations tend to throw some light on the function of the enzymes in the basal granules of the membranelles and the body cilia, even if only in a negative sense: if the enzyme were to appear just prior to membranelle formation, a role concerned with the mechanics of ciliary differentiation and structural maintenance might be tentatively ascribed to the enzyme. Since this, however, is not the case, the enzyme may possibly be involved in the energetics of ciliary motion.

In summary, the results tend to show that phosphatases in Stentor are fairly consistently present at definite loci of the cytosome, and that reorganization processes, unless they lead to the death of an animal or a fragment, are not correlated with significant changes in enzyme distribution. Newly differentiating organelles manifest characteristic enzymatic activity in parallel with morphological differentiation as such. The presence of phosphatases at circumnuclear sites may be significant in view of evidence (Weisz, 1949) that the macronuclear nodes discharge secretions (possibly phosphate-containing nucleic acid derivatives) into the endoplasm.

Summary

Phosphatase activity is studied in *Stentor coeruleus* by means of histochemical methods. "Alkaline" procedures are negative. Acid phosphatase may be consistently demonstrated in normal Stentors around the macronuclei, in the basal granules of the membranelles and the body cilia, in the endoplasmic fat vacuoles, and around the gastrioles. During starvation a gradual decrease in intensity and distribution of enzyme activity is observed, while in regeneration, physiological reorganization, and in vegetative division, activity remains unaltered in comparison to the normal animal. Presumptive evidence is obtained indicating that acid phosphatase in the basal granules is not primarily a factor in ciliary differentiation.

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THE PROTHORACIC GLANDS OF INSECTS IN RETROSPECT AND IN PROSPECT

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In 1762 the French anatomist, Lyonet, described as "granulated vessels" a pair of minute organs located within the thorax of caterpillars. This description was soon forgotten and for 187 years has been buried among the literature pertaining to insect anatomy. Meanwhile, within the twentieth century, the very same organs have been, and apparently continue to be, rediscovered by various investigators.

The resurrection of Lyonet's description of the "granulated vessels" seems particularly appropriate at the present time. For, within the past ten years, it has become increasingly evident that these organs, now known as "prothoracic glands," are among the most important endocrine glands in insects. The following remarkable paragraphs, which are quoted in translation, are therefore worthy of note, since they present not only the first, but to this day, the most complete description of the gross morphology of the new endocrine organs.

LYONET, P., 1762. Traité Anatomique de la Chenille Qui Ronge le Bois de Saule. Pages 435–437.

THE GRANULATED VESSELS

"Before going on to examine the fat-body and the parts which it encases, there remains to be described two strange vessels, which, on account of their small size, we did not mention in the general outline which we have given in Chapter VI on the interior parts of the caterpillar.

"These vessels, which, on account of their structure, will be called the granulated vessels, are located on the tracheae on the posterior side of the prothoracic spiracle where they form a semi-circle around trachea *a*. They pass dorsally along the tracheal branches *b* and *c*, the muscle *d*, and the cephalic tracheae, *e* and *f*. Each ends on its respective side between the cephalic tracheae *f* and *g*.

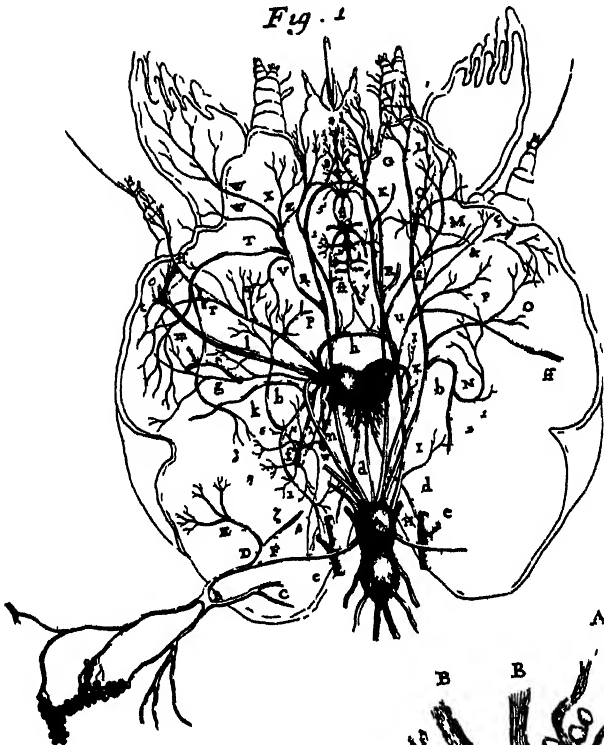
"They were consistently present in all the caterpillars of this species which I examined. On account of their small size they are difficult to distinguish easily without a magnifying glass, and they may be mistaken at first for a section of fat. When viewed through the microscope, they appear as they are represented in Pl. XII Fig. 8; in other words, like a long, narrow, irregular and curved mass of longish adherent grains of varying sizes, usually smaller in the direction of the superior line and at the points of insertion of the nerves and tracheae.

"When this mass of grains is dissected, it is found to be formed by a long membranous sac loaded with small blisters which open on it and which are filled, as is the sac, with white matter which presents nothing specific.

Nerves. "In the subject from which Fig. 8 was taken, the nerves A,A,A,A, which supply the granulated vessels seemed to me to come from the third and fourth branch and from the second subdivision of the second branch of the last pair of nerves from the second ganglion; those marked B,B, from the second pair of nerves from the third ganglion; and those marked C,C,C, from the first spinal connectives.

Tracheae. "The tracheae which insert here seemed to me to come from the first and from the second cephalic trunks, but I have neglected to examine, as should have been done, the tracheae and nerves of this small section.

Function. "I have not been able to discover the purpose of the granulated vessels. The relationship they have to the ovaries of some insects could cause them to be taken for real ovaries.



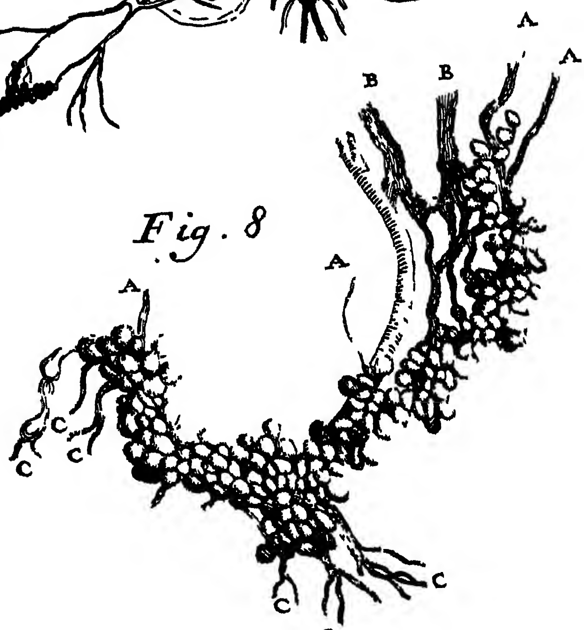


FIGURE 1. From Lyonet's Plate XII. Dissection of the head of the caterpillar as seen in ventral view. Note the prothoracic gland attached to its nerves in the lower left hand corner of the figure.

FIGURE 8. From Lyonet's Plate XVIII. Enlarged view of a prothoracic gland, showing innervation and tracheal supply.

But their location and form, which are very different from that of the ovary of the moth of this caterpillar, indicate the contrary. One might possibly suspect that they are the rudiments of the wings of this animal. This idea occurred to me first; but I was soon corrected when, upon opening a caterpillar on the point of changing into a pupa, I distinctly recognized the wings of the moth, and did not fail to find also the granulated vessels which had not changed shape. It is therefore only through studies of the anatomy of the pupa or the moth that one may hope to discover something on this point."

Nearly two centuries elapsed before application was made of the advice of the final paragraph. Then, in a series of investigations reported in 1940, 1941, and 1944, Fukuda was able to demonstrate that the prothoracic glands of *Bombyx mori* were involved in the endocrinological control of moulting, pupation, and adult development.

In the past few years certain of Fukuda's observations have been confirmed on other insects. In the Cecropia silkworm, for example, the development of the adult moth within the pupa requires a hormone secreted by the prothoracic glands (Williams, 1947, 1949a). But, in addition to this factor, the initiation of adult development also requires the *presence* of a second hormone secreted by the brain (Williams, 1946, 1947, 1949b) and the *absence* of a third hormone secreted by the corpora allata (Williams, 1949c).

Evidence of this type makes it increasingly clear that neither the prothoracic glands nor any other organ are endowed with the complete control over metamorphosis. In insects as in mammals, hormonal mechanisms seem to require a certain complexity in order to prove feasible and self-balancing.

From these considerations we are led to a somewhat more moderate evaluation of the role of the prothoracic glands than that which Fukuda has proposed. The prothoracic glands are but one component in an endocrinological system that presides over metamorphosis: among the other components in this system must be included at least two further organs, the brain and the corpora allata.

Notwithstanding this complication, the identification of the prothoracic glands of Lepidoptera as endocrine organs has provoked a re-examination of the endocrinology of metamorphosis and a search for comparable organs in other orders of insects.

The glands are now known to occur, not only in the Lepidoptera (Lee, 1948), but also in the Hymenoptera (Williams, 1948). Apparently homologous organs have been described as "prothoracic glands" in the Orthoptera (Scharrer, 1948), as "intersegmental organs" in the Odonata (Cazal, 1947, 1948; Deroux-Stralla, 1948a), and as "ventral glands" in certain other hemimetabolous insects (Pflugfelder, 1947). In the case of the Odonata the anatomical affinity between intersegmental organs and prothoracic glands has been reinforced by experimental evidence. Thus, according to Deroux-Stralla (1948b), the removal of the intersegmental organs results in abnormalities in moulting and suppression of adult development—effects that would be anticipated in terms of the proposed homology.

Since our knowledge of insect endocrinology is based so largely on studies of the Hemiptera, it is significant that organs anatomically identical with prothoracic glands have been found in nymphs of the lygaeid, *Oncopeltus fasciatus* (Edwards, 1948).

Of further interest is the description by Possompès (1946) of a pair of "peritracheal glands" in larvae of Chironomus. Possompès calls attention to the histo-

logical resemblance between these peritracheal glands in the lower Diptera and the lateral cells in the ring-gland of the higher Diptera. If the ring-gland should prove to contain a component homologous to the prothoracic glands of other insects, it would account for some of the peculiar endocrinological effects of this organ.

Thus, notwithstanding the fragmentary character of the present evidence, it seems probable that prothoracic glands and their homologues are widely distributed among insects. A more detailed evaluation of their role in development may lead to a sounder understanding of metamorphosis and may open to experimental attack certain facets of the problem that have hitherto remained inaccessible. In accordance with the advice of Lyonet, it is only through further study that we may hope to discover something on this point.

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THE EFFECTS OF ATMOSPHERIC PRESSURE AND COMPOSITION ON THE FLIGHT OF DROSOPHILA

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In the absence of a satisfactory theory of minute airfoils, not to mention airfoils which like the insect wing undergo continuous and variable angular motion, only limited help in the detailed analysis of insect flight can be obtained at present from the science of aerodynamics. Nevertheless, it is worthwhile to attempt to apply certain elementary aerodynamic concepts in an effort to develop a rational basis for the study of the flight process.

One such approach is to regard the wings of an insect as diminutive paddles which in each wingbeat serve to impart a specific average velocity to a specific mass of air. This point of view has already proved useful in analyzing the correlation between frequency of wingbeat and the dimensions of the wings and thoracic muscles in various species of Drosophila (Reed, Williams and Chadwick, 1942). Considering flight of an insect in these simple terms, the power output (P) should be proportional to the mass of air moved per beat (m), the square of the average velocity (v) imparted to this mass, and the wingbeat frequency (f):

$$P \propto mv^2f. \tag{1}$$

For present purposes the relationship may be further simplified, since

$$v \propto f$$

and, therefore, substituting in Equation (1),

$$P \propto mf^3,$$

or

$$f^3 \propto P/m. \tag{2}$$

Thus, according to this analysis, the cube of wingbeat frequency should be proportional to the power output divided by the mass of air moved per beat. That this relationship has real validity is indicated by correlations already demonstrated between wingbeat frequency and the energy consumption during flight. In the case of Drosophila the flight energy (oxygen consumption and carbon dioxide production) was found actually to vary as the cube of wingbeat frequency (Chadwick and Gilmour, 1940; Chadwick, 1947). Manifestly, such a correlation would be expected on the basis of Equation (2), provided that the mass of air moved in each remained constant.

While the basic relationship has then been verified experimentally, the effects of varying the mass of air moved per beat have in contrast remained very largely a matter of speculation. V. Buddenbrock (1919), Roch (1922) and Sotavalta (1947) adopted the simplest means of altering this factor by clipping the tips from the wings. Though these investigators have offered various interpretations of their results, the increase in wingbeat frequency they observed seems to us attributable directly to the decreased mass of air moved per beat. However, the quantitative aspects are not readily established in experiments of this sort, which we have also performed, for the reasons that surfaces of equal area from different parts of the wing may not be equivalent aerodynamically and that we have as yet no means of measuring their presumably different contributions to the outflowing air stream. Thus, while one may observe that progressively shortening the wings results in a progressive increase in the rate of wingbeat, one is unable to derive a precise statement of the relationship thereby revealed. Such experiments suffer also, of course, from the necessity of mutilating the structure one is attempting to study.

Fortunately these difficulties can be avoided relatively easily, since, if the wings are regarded as sweeping out a specific volume of air with each beat, the mass of air moved is obviously dependent on the gaseous density of the medium. By varying the density, the wings may be made to sweep out a volume whose mass may be altered continuously, and the resulting changes in performance correlated, in terms of wingbeat frequency, with the density change. Alterations of density are produced and measured conveniently merely by varying the pressure of the air in which the insect flies. Our problem resolves itself then into an examination of the effects of changes in atmospheric pressure on the rate of wingbeat.

As far as we are aware, there has been no adequate investigation of this matter. Magnan (1934) states that "frequency changes with pressure also. Thus a fly making 160 strokes per second makes 20 more when placed in a vacuum corresponding to an altitude of 2000 meters." By an acoustic method Sotavalta (1947) determined the wingbeat frequencies of several species of bees at a series of subatmospheric pressures, but observed no deviations from the rates measured under normal conditions. Case and Haldane (1941) note in passing that Drosophila, exposed to an air pressure of 10 atmospheres, was unable to fly. These limited observations are all we have been able to find in the literature.

Our initial and simple objective of studying the effects of variation in atmospheric pressure has, as is so frequently the case, raised more problems than were contemplated at the outset. For, in addition to altering density, variations in air pressure produce systematic changes in oxygen tension and in pressure as such. On this account, efforts to study each of these factors separately were necessary. Measurements of the oxygen consumption during flight at normal and reduced pressures were also made when it became apparent that, for an understanding of the other data, more information was needed about the energy relationships concerned.

Materials and Methods

A. Measurements of wingbeat frequency

Our first experiments were performed on *Drosophila repleta* Wollaston, a species particularly adapted to these studies on account of its dependable tarsal flight

reflex. Later, *D. virilis* Sturtevant was also used. In working with *D. repleta,* individuals were selected at random from a wild population that maintained itself in the animal rooms and were used without regard to age or sex, since we were unaware at the time that frequency of wingbeat is determined to some extent by these variables. This defect in technique has contributed to the scatter in the data, and was avoided in the studies with *D. virilis,* which were grown under standard conditions at 25 degrees C. and isolated daily on emergence.

The apparatus in which wingbeat frequency was measured is a simplified version of the flight chamber described by Williams and Chadwick (1943), and has been diagrammed in Figure 1. It consists of a glass pressure chamber whose temperature was controlled either by circulation of water through a surrounding jacket or by immersion in a constant temperature bath. Provisions were made for clamping

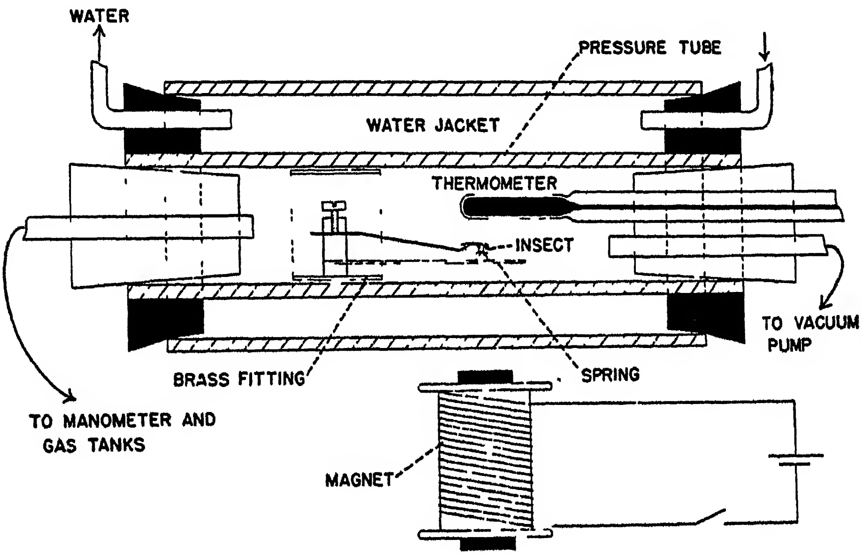

FIGURE 1. Apparatus for measurement of wingbeat frequency at various pressures. For explanation see text.

rubber stoppers, one of which held a thermometer, into the ends of the pressure chamber, while glass tubes, passing through the stoppers, allowed gas mixtures of known composition to be circulated.

These mixtures were made up at high pressure in commercial gas cylinders and analyzed before use. At each change of gas mixture the chamber was washed thoroughly with the succeeding mixture. Pressure within the chamber was varied by the addition of compressed gases or by means of a vacuum pump. A pressure gauge and mercury manometer, measuring up to 5 atmospheres, were sealed into the gas lines and permitted a continuous check on the pressure within the experimental chamber. Relative humidity was held at or near 100 per cent by placing a few drops of water within the chamber and by bubbling the gases through water as they entered.

The rate at which pressures were altered between successive sets of measurements had no obvious effect on wingbeat frequency. Ordinarily these changes were made fairly quickly and though several minutes were then allowed for equilibration even this period of adaptation appeared unnecessary. It was possible therefore to test the various pressures in rapid random succession.

All the data obtained with *D. repleta* have been computed in terms of the response at 25 degrees C. In some of the earlier experiments the measurements were made at temperatures that deviated slightly from 25 degrees C.; these have been adjusted by applying a factor derived from studies of the effect of temperature on wingbeat frequency. Although the validity of this treatment was tested and confirmed, it became unnecessary in all later experiments when the flight chamber was maintained at 25 ± 0.1 degrees C. With *D. virilis,* various constant temperatures were used, as stated in the tabulation of results.

Measurements of wingbeat frequency were made on fastened specimens according to the method previously described. To evoke and terminate the flight of the insect within the sealed chamber, the tarsal reflex was utilized. Flight was induced by withdrawing a spring platform from under the animal's feet and stopped by interrupting the current to the electromagnet shown in Figure 1.

Frequency of wingbeat was measured by means of a General Radio "Strobotac." In each instance, the maximal frequency, occurring within the first few seconds of flight, was recorded. Each flight was therefore extremely brief, with a duration in most cases of about 2 seconds. In this way the onset of fatigue was postponed, so that several hundred measurements could be made on most individuals. Determinations were made at intervals of 10 seconds, the 8 seconds of rest between flights having been found adequate for recovery. The response of each individual to each experimental condition was usually recorded as the mean of 20 measurements.

Each fly was tested initially in air at atmospheric pressure and then under a variety of experimental conditions. At intervals during an experiment the performance was rechecked in air; any animal showing significant deviation from its initial response was discarded. Since it was impossible to test all individuals under all circumstances and since there were considerable differences in the wingbeat frequencies of different individuals in air at 760 mm. Hg (mainly because of our ignorance of the influence of age and sex), results for *D. repleta* have been calculated in terms of the deviation in frequency under each set of conditions from the frequency observed for that individual in air at 760 mm. Hg. For presentation of the average data as in Tables 1, 3, and 6, the average deviations in wingbeat frequency at each pressure have been added to or subtracted from the mean frequency for all individuals at 760 mm. Hg in air, in order to provide a more direct comparison of the rates in the various media used. Thus, for example, the average frequency of 10,700 cycles per minute at 3860 mm. Hg shown in Table 1 was obtained by subtracting 1990, the average decrement for these 17 animals from their rates at 760 mm., from 12,690, the mean for all 72 flies at normal pressure. The statistically preferable procedure of using a different randomly selected sample of flies for each set of conditions would have been impractical, particularly since we had not then succeeded in establishing *D. repleta* in culture.

With *D. virilis*, a series of only 4 or 5 pressures was used, and each insect was flown 10 times at each pressure. The results thus obtained were averaged and are presented in this form in Table 2.

B. Measurements of oxygen consumption during flight

Details of the technique for measuring oxygen consumption of Drosophila in flight have been described in previous reports (Chadwick and Gilmour, 1940; Chadwick, 1947). In the present study, differential volumeters (Fenn) were used and an arrangement adopted which allowed simultaneous evacuation of both vessels, as shown in Figure 2. The vessels had capacities of about 13 ml. and were connected by a capillary with a volume of about 5 cu.mm. per cm.

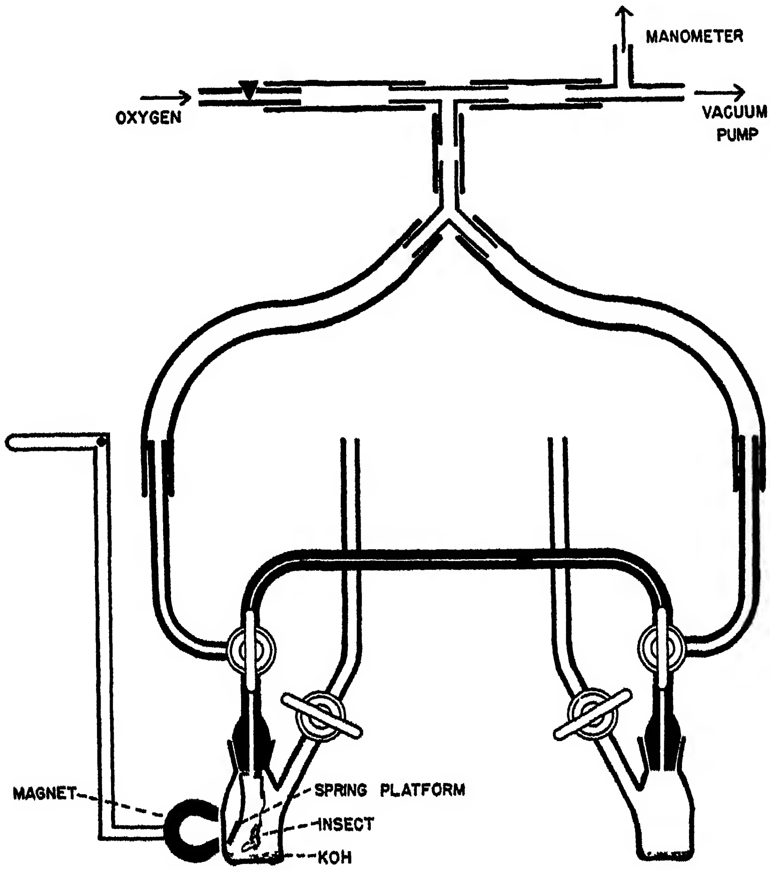

FIGURE 2. Apparatus for measurement of oxygen consumption during flight at normal and reduced pressures. For explanation see text.

Individual *D. virilis* of known age were anesthetized with carbon dioxide gas and fastened with paraffin to a fine wire which was then attached to the head of the respirometer. The fly was suspended head-down in the vessel, its feet in contact with the usual retractible platform. To depress the paraffin-coated platform and induce flight, a small permanent magnet was brought up to the side of the vessel in the bath. Carbon dioxide given off by the insect was absorbed in 0.1 ml.

of 15 per cent KOH in the bottom of the vessel, which had been fitted with a sleeve of filter paper to increase the absorbing surface.

During a 20-minute period of equilibration at 19.3 ± 0.01 degrees C. in the water bath, both vessels were gassed out with oxygen from a commercial cylinder. The stopcocks were then turned to running position and the resting oxygen consumption measured for 30 minutes or longer. In about half the experiments, flight was then induced at normal pressure and allowed to continue for 10 or 20 minutes, during which time readings of oxygen consumption were taken every minute and measurements of the rate of wingbeat every 10 seconds. For the latter, the specimen was viewed in silhouette against the flash lamp, which had been let down into the bath

TABLE 1

Wingbeat frequency as a function of air pressure and density
Drosophila repleta in moist air at 25° C.

Air pressure mm. Hg	Density gms./liter	Average wingbeat frequency beats/min.	Number of specimens	Number of measurements
3860	6.00	10,700	17	322
3450	5.36	10,910	22	442
3100	4.82	10,990	25	525
2820	4.38	10,940	21	430
2580	4.01	11,330	20	425
2320	3.60	11,330	20	410
2100	3.26	11,660	21	425
1880	2.93	11,570	15	270
1660	2.57	11,720	13	224
1380	2.14	12,050	13	216
1200	1.86	12,110	14	250
980	1.51	12,320	15	310
760	1.17	12,690	72	2730
680	1.05	12,880	22	399
600	0.92	12,930	20	358
500	0.77	13,070	20	390
400	0.61	13,210	37	770
300	0.45	13,310	20	353
260	0.39	13,540	19	824
200	0.30	13,680	32	615
140	0.20	13,950	18	347
100	0.14	14,060	14	237

inside a glass cylinder. After the measurements at normal pressure, the system was evacuated to 200 or 400 mm. Hg, and the same procedure repeated. In the remaining experiments, the order of pressures was reversed; for example, the first set of measurements was made at 200 or 400 mm. Hg, and the second at normal pressure.

Careful attention to the lubrication and seating of stopcocks and other joints was essential since an inward leak amounting to a fraction of a cu.mm. per minute could render the measurements of oxygen consumption at low pressures valueless. Vaseline was used successfully as a stopcock grease at temperatures of 20 degrees C. or less, but although this and several other lubricants were tried, attempts to repeat

these experiments at 26 degrees C. failed because leaks around the stopcocks invariably developed before a run could be completed. Only those experiments were considered valid in which a reasonably constant rate of resting oxygen consumption, of a reasonable magnitude in comparison with earlier measurements, was obtained for at least one half-hour before flight at each pressure, and in which the rate of oxygen consumption returned to and maintained a value approximating the preflight level within a few minutes after flight had ceased.

Although it was impossible in the system diagrammed to be certain that leakage was zero, one can state with assurance that any leaks which did occur were not greater than the average resting rate of about 30 cu.mm. per gm. per minute. Since the flight oxygen consumption was computed by subtracting the resting rate from the total measured during the flight which followed immediately, such errors would

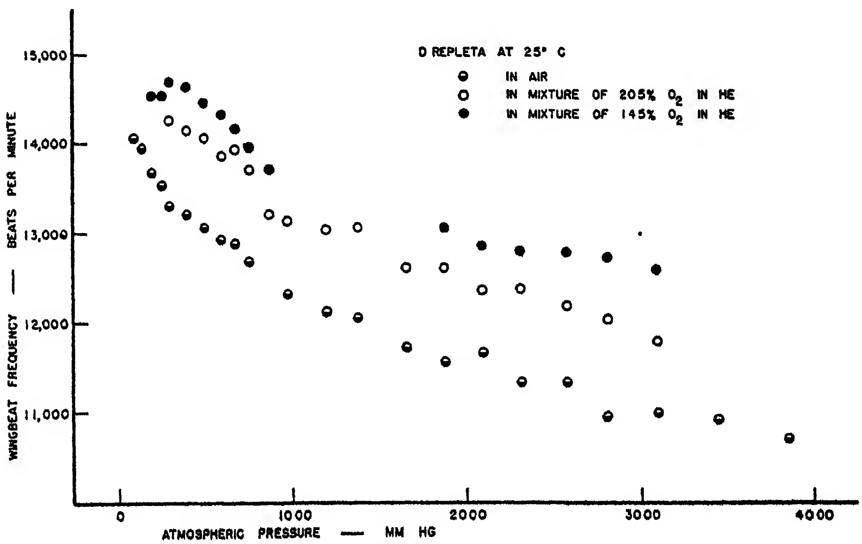

FIGURE 3. Wingbeat frequency of *D. repleta* as a function of atmospheric pressure in air and in two oxygen-helium mixtures.

affect mainly the resting rates rather than the flight respiration in which we were chiefly interested. However, if one makes the highly conservative allowance of a possible error of 30 cu.mm. per gm. per minute, this will amount only to some 8–9 per cent of the average flight oxygen consumption measured at 19.3 degrees C. and will not alter significantly the conclusions we have drawn from the data.

Difficulty was experienced in some of the early attempts in obtaining a flight response from the insect after the vessels had been lowered into the bath. Illuminating the experimental vessel with a 40-watt bulb in a reflector at the side of the bath overcame this trouble. The lamp neither interfered with the observations of wing movement nor, since the glass wall of the bath and at least 6 inches of well-stirred water separated it from the respirometer, disturbed the measurements of oxygen consumption. In blank runs, no movement of the index drop occurred as a result of turning the lamp or stroboscope on or off.

Results

A. Observations of wingbeat frequency

In order to have a means of differentiating between effects due to variation in total pressure, oxygen tension and gaseous density, *D. repleta* was tested in 5 different media: (1) air, (2) 14.7 per cent oxygen in nitrogen, (3) nitrogen-oxygen mixtures of higher oxygen content than air, (4) 14.5 per cent oxygen in helium and (5) 20.5 per cent oxygen in helium. The rate of wingbeat of *D. virilis* was measured in air at 5 different pressures, both at 19.3 degrees C and at 25.9 degrees C.; and at normal pressure, 25.9 degrees C., in a mixture of 6.1 per cent oxygen in nitrogen.

TABLE 2

Wingbeat frequency of D. virilis as a function of air pressure

Pressure in mm Hg		1520	760	400	200	100
Specimen number	Age in days	Wingbeat frequency in beats per minute				
a. Moist air at 19.3 degrees C.						
3–112 ♀	8–9	10,690	11,000	11,680	12,660	13,180
3–113 ♀	8–9	8,740	9,550	10,390	11,280	refused
3–151 ♀	12–13	11,120	11,470	12,060	12,980	refused
3–152 ♀	12–13	10,410	11,100	11,930	12,770	refused
3–153 ♀	12–13	10,100	11,150	11,650	12,220	refused
3–155 ♀	12–13	10,570	11,290	12,300	13,390	14,020
3–157 ♀	12–13	10,630	10,870	11,620	12,560	refused
3–158 ♀	12–13	9,210	10,380	10,630	11,500	refused
3–159 ♀	12–13	10,950	11,120	11,720	12,030	13,060
3–1512 ♀	12–13	11,030	11,390	11,960	12,750	13,380
Average (10 flies)		10,350	10,930	11,590	12,410	—
Average (4 flies which flew at 100 mm.)		10,810	11,200	11,920	12,710	13,410
b. Moist air at 25.9 degrees C.						
3–211 ♀	4–5	13,710	14,010	14,870	15,670	16,370
3–212 ♀	4–5	13,870	14,290	14,770	15,590	16,130
3–213 ♀	4–5	14,250	14,510	14,810	15,670	15,790
3–214 ♀	4–5	13,650	13,990	14,290	14,650	15,150
3–215 ♀	4–5	13,450	14,050	14,710	15,550	15,970
3–217 ♀	4–5	14,170	14,590	15,290	16,010	16,250
3–218 ♀	4–5	13,790	14,210	14,930	15,670	15,850
3–221 ♀	5–6	12,050	12,880	14,090	15,510	refused
3–222 ♀	5–6	12,970	13,590	14,530	15,630	16,050
3–223 ♀	5–6	10,320	10,950	12,330	13,370	13,940
Average (10 flies)		13,250	13,710	14,460	15,330	—
Average (9 flies which flew at 100 mm.)		13,350	13,800	14,500	15,310	15,720

Each observation is the mean of 10 measurements.

1. Air

The effects of variation in air pressure on the frequency of wingbeat of *D. repleta* were studied in a total of 72 individuals, over a range of 5 atmospheres. As indicated in Table 1 and Figure 3, the frequency of wingbeat decreased gradually as the pressure increased. This effect was observed over the whole range of pressures investigated, from 80–100 mm. Hg, below which the animals failed to fly when stimulated, to a pressure of 3860 mm. Hg.

Examination of the data reveals an apparent discontinuity in the relationship at about 680 mm. Hg. This is best visualized on the logarithmic grid of Figure 4. Our reasons for considering it an artefact are given in the discussion. No such discontinuity is evident in the data obtained at two temperatures with *D. virilis* (Table 2 and Figure 4).

TABLE 3

Wingbeat frequency as a function of pressure in a mixture of 14.7 per cent oxygen in nitrogen Drosophila repleta at 25° C.

Total pressure mm. Hg	Density gms/liter	Average wingbeat frequency beats/min.	Number of specimens	Number of measurements
3100	4.78	10,950	12	202
2820	4.34	11,090	7	140
2320	3.57	11,480	8	87
1880	2.89	11,610	12	190
1380	2.12	11,960	18	290
980	1.50	12,220	19	370
760	1.16	12,600	39	1197
680	1.04	12,860	12	170
600	0.91	12,890	5	70
500	0.76	13,300	11	157
400	0.60	13,260	9	140
300	0.45	13,540	10	146
200	0.30	13,970	4	55

2. 14.7 per cent oxygen in nitrogen

A further series of 39 *D. repleta* was tested in a mixture of subnormal oxygen content in order to emphasize the possible effects of decreased oxygen tension at low total pressures. As shown in Table 3 and Figure 4, no significant difference was evident in comparison with the relationship observed in air. Comparative data for *D. virilis* in air and in 6.1 per cent oxygen in nitrogen are given in Table 4. Again no difference was observed.

3. Atmospheres of high oxygen content

By somewhat different measures the effects of subatmospheric pressures of mixtures with high oxygen content were studied. In these experiments each individual was tested at a specific low pressure in air and then subjected to the same pressure in an atmosphere rich in oxygen. The two sets of measurements, samples of which are given in Table 5, showed no significant differences. It was never possible to

cause the frequency of wingbeat to rise above the value in air by supplying a greater than normal proportion of oxygen.

4. *Helium-oxygen mixtures*

Having found no correlation between the tension of oxygen and the response of the insect to pressure, there remained the problem of distinguishing between the two other variables involved in these experiments; namely, gaseous density and pressure *per se*. Their separation seemed difficult at first, since the density and total pressure of a given gas mixture are directly proportional. However, the fact that helium is an inert gas with a density only about one-seventh that of nitrogen offered a means of attacking the problem. Using helium and oxygen, mixtures

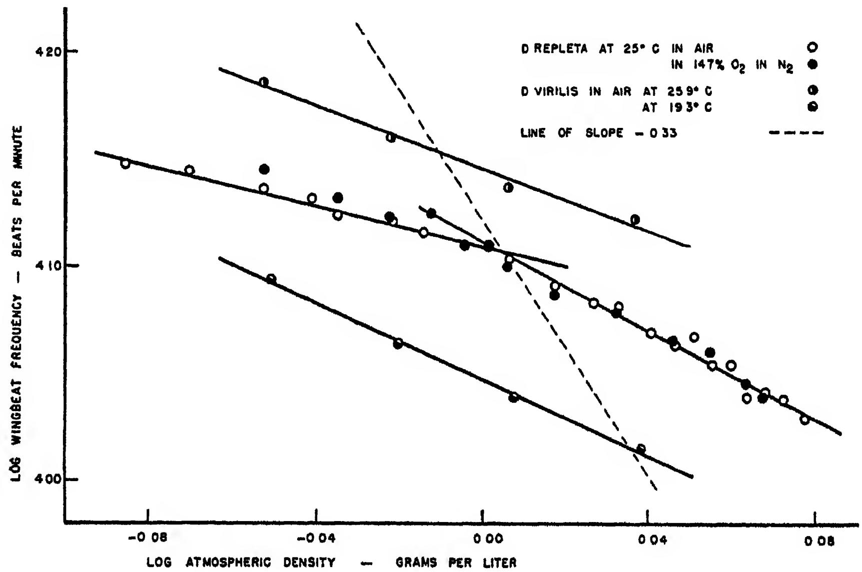

FIGURE 4. Wingbeat frequency of *D. repleta* and *D. virilis* as a function of atmospheric density. Solid lines fitted to empirical data by the method of least squares. Broken line of slope — 0.33 added for comparison.

may be prepared which differ from air or other oxygen-nitrogen mixtures in density but not, presumably, in regard to most other properties which are physiologically significant.

Two helium-oxygen mixtures were used for this purpose, one containing 14.5 per cent oxygen, the other 20.5 per cent. At 25 degrees C. and one atmosphere, the densities of these mixtures, both of which contained water vapor and small amounts of nitrogen, were approximately 0.33 and 0.39 grams per liter, respectively, as compared with 1.17 grams per liter, the density of moist air.

Frequency of wingbeat was measured with *D. repleta* in each of these mixtures throughout most of the range of pressures with results recorded in Table 6 and Figure 3. It is evident that the frequency was higher in either mixture than in

TABLE 4

Wingbeat frequency of D. virilis as a function of oxygen tension
Moist gas at 25.9 degrees C. and 760 mm. Hg

Specimen number	Age	Wingbeat frequency in	
		Air	6.1 per cent O_2 in N_2
	days	beats per minute	
3-214 ♀	4-5	13,240	13,220
3-215 ♀	4-5	13,300	13,180
3-217 ♀	4-5	13,840	13,940
3-218 ♀	4-5	13,460	13,120
3-221 ♀	5-6	12,900	12,900
3-222 ♀	5-6	13,700	13,820
3-223 ♀	5-6	10,740	10,690*
3-232 ♀	6-7	12,670	12,710
3-234 ♀	6-7	11,730	11,950
3-235 ♀	6-7	13,140	13,210
Average (10 flies)		12,870	12,870

* Rate of this specimen apparently depressed by previous flights; initially the rate was 10,950 in air at 760 mm. Hg (Table 2).
Each observation is the mean of 10 measurements.

air of the same pressure, and that it was highest at any given total pressure in the mixture having the least density.

During these experiments the animals reacted badly to the helium mixtures. It was frequently difficult or impossible to induce flight with the usual stimulus, and, after a relatively small number of flights, the wingbeat frequency began to decrease. The behavior resembled that of a fatigued animal in air, and on this account it was

TABLE 5

Wingbeat frequency in air and in atmospheres with greater oxygen content
Drosophila repleta at 25° C.

Specimen number	Pressure	Wingbeat frequency in		Oxygen content of mixture
		Air	O_2-N_2 Mixture	
	mm. Hg	beats/min.	beats/min.	per cent
97	1660	13,060	12,590	100
87	680	14,310	14,000	100
93	600	12,060*	11,980	100
90	400	13,430	13,390	44
97	200	14,440	14,440	100
67	200	15,080	14,370	50
64	100	16,380	15,870	50

* In 3.9 per cent oxygen in nitrogen.
Each frequency datum is the mean of 20 observations. Atmospheres were saturated with water vapor at 25° C.

necessary to reduce the number of flights. Thus the points determined for individual specimens during this series of experiments have been averaged as a rule from only five measurements at each pressure. Specimens which had become re-

TABLE 6

Wingbeat frequency as a function of pressure and density in helium-oxygen mixtures Drosophila repleta at 25° C.

Total pressure mm. Hg	Density gms /liter	Average wingbeat frequency beats min.	Number of specimens	
a. 14.5 per cent oxygen in helium				
3100	1.34	12,590	10	50
2820	1.22	12,730	9	45
2580	1.12	12,780	9	45
2320	1.00	12,800	8	40
2100	0.91	12,860	8	40
1880	0.81	13,070	8	40
880	0.38	13,720	9	45
760	0.33	13,960	15	75
680	0.29	14,160	5	25
600	0.26	14,330	5	25
500	0.21	14,460	7	35
400	0.17	14,640	7	35
300	0.13	14,680	6	30
260	0.11	14,530	5	25
200	0.08	14,530	5	25
b. 20.5 per cent oxygen in helium				
3100	1.62	11,780	7	50
2820	1.47	12,030	7	50
2580	1.35	12,190	8	55
2320	1.21	12,390	7	40
2100	1.10	12,370	7	35
1880	0.98	12,610	8	40
1660	0.87	12,610	7	45
1380	0.72	13,070	8	35
1200	0.62	13,040	7	35
980	0.51	13,140	9	45
880	0.46	13,210	8	40
760	0.39	13,710	22	135
680	0.35	13,930	8	40
600	0.31	13,870	10	50
500	0.26	14,060	11	55
400	0.21	14,150	9	45
300	0.15	14,260	7	35

fractory in the helium mixtures resumed a normal behavior when returned to air. It was observed also that the animals responded more readily in the helium mixtures when the total pressure was high than when it was one atmosphere or less.

5. Other observations

a. Humidity. In an early series of experiments we found that the frequency of wingbeat failed to increase at subatmospheric pressures when the relative humidity within the flight chamber was low. On the contrary, the rate decreased rapidly and the specimens soon became incapacitated. This effect seems explicable in terms of damage to the insect from loss of water; possibly this factor may account for the negative results reported by Sotavalta (1947).

b. Stroke amplitude. During measurements at high pressures a reduction in the stroke amplitude was evident in most individuals. Though the magnitude of

TABLE 7

Wingbeat frequency of D. repleta before and after removal of halteres

Specimen number	Wingbeat frequency in beats per minute after treatment indicated		
	Etherized and mounted	Re etherized	Again re-etherized and halteres removed
48 ♀	10,220	10,190	10,250
49 ♂	9,640	9,800	9,930
50 ♂	9,950	10,130	9,890
54 ♀	10,660	10,670	10,490
55 ♀	10,340	10,340	10,380
56 ♀	12,290	12,240	12,280
57 ♀	12,360	12,350	12,120
58 ♀	10,880	10,930	10,610
59 ♀	11,510	11,600	11,720
60 ♀	11,850	11,940	11,880
61 ♀	10,520	10,540	10,380
62 ♀	11,110	11,090	11,580
64 ♂	10,160	10,260	10,320
65 ♀	11,250	11,220	11,450
66 ♀	10,360	10,530	10,520
67 ♀	10,320	10,340	10,640
68 ♀	11,150	11,120	11,210
Average	10,850	10,900	10,920
Standard error	±194	±180	±188

Each datum is the average of 20 determinations. The experiments were run in moist air at 20° C. and 615 mm. Hg.

these changes was not measured, they are of considerable importance theoretically, as will be brought out in the discussion.

c. Halteres. An important rôle of the halteres in regulating the wingstroke has been proposed frequently in the past and reemphasized recently by Pringle (1948), so that it seemed advisable to give some attention to these organs under the conditions of our experiments. As originally reported by Williams and Reed (1944) and subsequently confirmed by Pringle (1948), the halteres are vibrated during flight at the same frequency as the wings, but in opposite phase. See also Curran (1948). This synchrony persists at all pressures. Furthermore, wingbeat frequency in air at various pressures was unaffected by amputation of the halteres as

illustrated by the example given in Table 7. Even when the rate had been increased by clipping the wings, amputation of the halteres had no further effect (Table 8). The relationship between pressure and wingbeat frequency was apparently identical in the case of normal and haltereless flies; in fact, the only difference we have seen in the wing action of the two groups is in the somewhat steadier wingbeat frequency of haltereless flies during continuous flight.

d. Failure to respond at low pressures. In experiments at reduced pressure it was never possible to evoke flight when the total pressure was less than 80 to 100 mm. Hg. Of this pressure approximately 24 mm. Hg must be assigned to water vapor so that, in air, the partial pressure of oxygen amounted only to some 12 to 16 mm. Hg. Under such circumstances one might suppose that failure to fly was due to oxygen lack. Yet flight at lower pressures was still unobtainable when pure

TABLE 8

Wingbeat frequency of D. repleta before and after clipping wings and removing halteres

Specimen number	Wingbeat frequency in beats per minute after treatment indicated				
	Etherized and mounted	One wing clipped	Both wings clipped	Re-etherized	Again re-etherized and halteres removed
34 ♂	12,230	12,980	14,170	14,050	14,010
37 ♀	12,680	13,520	14,930	15,440	15,150
38 ♀	11,650	12,200	12,560	12,680	12,910
39 ♀	11,880	12,880	13,360	13,260	13,460
40 ♀	11,460	12,590	12,810	12,900	13,310
41 ♂	11,340	12,120	12,730	12,710	12,810
43 ♂	11,600	11,800	12,330	12,400	12,150
Average	11,850	12,580	13,270	13,750	13,400

Each datum is the average of 10 determinations. The experiments were run in moist air at 20° C. and 615 mm. Hg, except for those with Specimen Number 34, which were run at 22° C. and 645 mm. Hg. The observations are in contradiction with the finding of Roch (1922) that clipping one wing leaves the wingbeat frequency unaltered. Particular care was taken to cut the same amount from each wing, as nearly as possible; in general, from one-quarter to one-half the wing was removed, by a transverse cut.

oxygen was substituted for air. This rather surprising observation was verified repeatedly.

e. Limiting tension of oxygen. Although some factor other than oxygen lack appears to prevent a flight response at total pressures below about 80 to 100 mm. Hg, there is also a lower limit to the oxygen tension consistent with brief interrupted flight. This is usually encountered when the oxygen tension in the gas mixtures is reduced below 15 to 20 mm. Hg. Thus in the case of air the limitation due to lowering the partial pressure of oxygen is about the same as that imposed by the unknown factor noted in the previous paragraph.

B. Measurements of oxygen consumption

Respiratory rates averaged over 10 or 20 minutes of continuous flight at normal and reduced pressure, together with the average rates of wingbeat observed simul-

TABLE 9

Oxygen consumption and wingbeat frequency of D virilis during continuous flight at normal and reduced pressures in oxygen

Specimen number	Age	Weight	At 400 mm Hg		At 760 mm. Hg		Ratio
			Frequency	Oxygen consumption	Frequency	Oxygen consumption	$\frac{O_2 \text{ at } 400 \text{ mm. Hg}}{O_2 \text{ at } 760 \text{ mm. Hg}}$
	days	mg.	beats per minute	cu. mm per gm per minute	beats per minute	cu. mm per gm per minute	
*2–181 ♀	8–9	2.51	10,760	341	10,340	310	1.10
*2–182 ♀	8–9	2.72	11,060	337	10,460	306	1.10
*2–211 ♀	12–13	2.22	11,020	404	10,710	391	1.03
*2–221 ♀	6–7	2.39	10,770	401	10,360	406	0.99
2–231 ♀	7–8	2.69	10,210	337	9,580	312	1.08
2–251 ♀	9–10	1.93	11,050	248	9,520	236	1.05
2–252 ♀	9–10	2.79	10,160	330	9,410	330	1.00
2–281 ♂	4–5	1.64	10,400	363	9,140	356	1.02
Average		2.36	10,680	345	9,940	331	1.05
			At 200 mm. Hg		At 760 mm Hg		$\frac{O_2 \text{ at } 200 \text{ mm Hg}}{O_2 \text{ at } 760 \text{ mm Hg}}$ Ratio
3–11 ♀	5–6	2.17	12,220	263	10,450	315	0.83
3–12 ♂	5–6	1.60	12,480	467	11,270	473	0.99
3–21 ♀	6–7	1 63	12,740	348	10,120	266	1.31
3–71 ♂	3–4	1.64	11,770	377	10,080	441	0.85
*3–72 ♀	3–4	1.90	12,050	345	10,200	409	0.84
*3–81 ♀	5–6	1.93	12,040	289	10,340	333	0.87
*3–91 ♀	6–7	2.25	10,910	310	9,670	320	0.97
*3–92 ♀	6–7	2.74	11,670	343	10,480	358	0.96
*3–101 ♀	7–8	1.84	11,210	308	10,270	294	1.05
Average		1.97	11,900	339	10,320	356	0.96

* Specimens thus marked were flown first at normal pressure; the others were flown first at reduced pressure.

Volumes corrected to NTP.

taneously, are presented in Table 9. Since the purpose of these measurements was to learn what effect alterations in density might have on the output of power by the flying insect, the runs were made in an oxygen atmosphere. As indicated above, the initial frequency of wingbeat appears to be independent of oxygen tension, but this is not true of the frequencies observed during continuous flight. In atmospheres with a low partial pressure of oxygen, the rate of wingbeat decreases rapidly after the first few seconds, and flight is maintained for a shorter period than under normal conditions. The rate of oxygen consumption also is depressed (Chadwick and Gilmour, 1940; Davis and Fraenkel, 1940). In the present experiments with *D. virilis* the tension of oxygen was higher than that of moist air at normal pressure even in the runs at a total pressure of only 200 mm. Hg, so that the results may be considered merely in reference to density change.

The flights were held to relatively short durations in order to minimize complications due to progressive fatigue, which reduces the rate at which oxygen is consumed. This factor was further equalized in the averages by reversing the order of pressures used in half the cases. The results thus obtained at 200 mm. Hg and 400 mm. Hg did not show any significant change in the average rate of oxygen consumption during flight at these pressures in comparison with the performance of the same individuals at 760 mm. Hg, although the data at 200 mm. Hg indicate a possibly significant depression for 4 of the 9 flies tested.

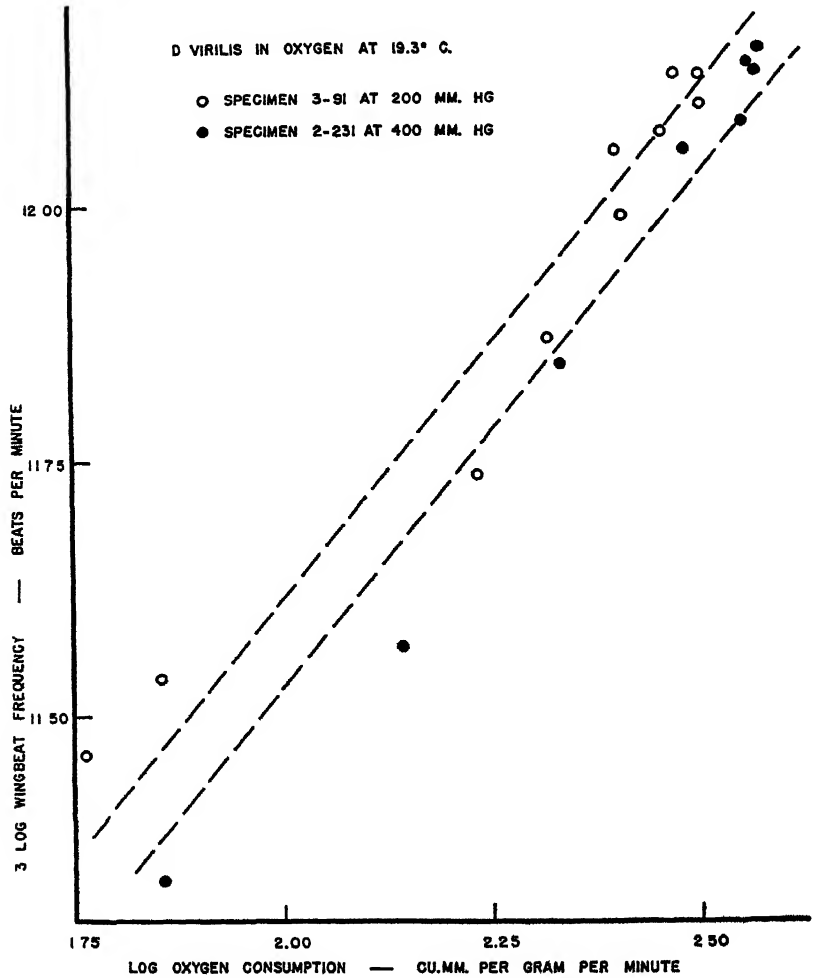

FIGURE 5. Correlation between wingbeat frequency and rate of oxygen consumption during flight in an oxygen atmosphere at reduced pressures. The broken lines have been drawn to conform to the equation, $3 \log F = \log k + \log O_2$, by using for $\log k$ the average value obtained for each specimen when the paired empirical values of wingbeat frequency and oxygen consumption were substituted into the above relationship. Each point represents the average values observed during 5 minutes of continuous flight.

The average oxygen uptake for 22 specimens, including some for which satisfactory runs were not obtained at reduced pressure, was 357 cu.mm. per gm. per minute at 19.3 degrees C. and 760 mm. Hg. These data agree closely with rates reported previously for flights of comparable duration with this species in air at 20 degrees C. (Chadwick, 1947). Thus it is apparent that the rate of oxygen consumption was not increased by supplying oxygen in excess of the tension normally present at one atmosphere.

In earlier studies a proportionality between the cube of wingbeat frequency and the rate at which oxygen is consumed (or CO_2 produced) was demonstrated for flights in air at normal pressure. Here, a few additional flights of about 40 minutes duration were made at 200 and 400 mm. Hg, and from them it was ascertained that the relationship

$$3 \log f = \log K + \log O_2 \tag{3}$$

applies at pressures other than normal. Typical results of such runs have been plotted in Figure 5.

Discussion

The measurements reported above demonstrate that the principal factor concerned in the relationship between wingbeat frequency and atmospheric pressure is variation in gaseous density. A comparison of the values obtained in the helium

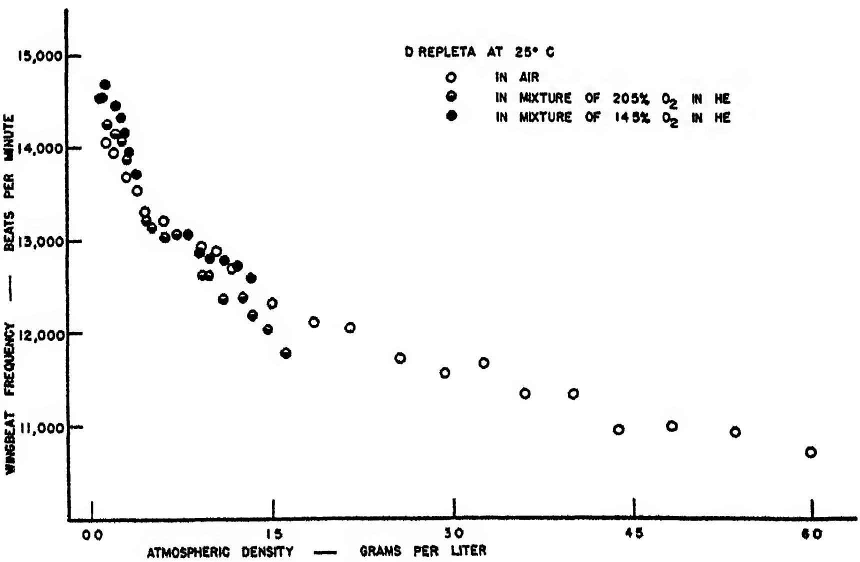

FIGURE 6. Wingbeat frequency of *D. repleta* as a function of atmospheric density in air and in two oxygen-helium mixtures.

mixtures with those obtained in air shows this clearly. In Figure 6 the three sets of data have been plotted together on coordinates where density, rather than total pressure, is the independent variable. Considering that the helium mixtures had

detrimental effects upon the animals, and that there are in the curves irregularities due to various factors other than density, the agreement is convincing. Thus, at densities of about 1 gm. per liter, the average wingbeat frequencies in all the gas mixtures were identical within 2 per cent, although the determinations were made at less than one atmosphere in air, approximately 2½ atmospheres in the 20.5 per cent oxygen in helium mixture, and at over 3 atmospheres in the 14.5 per cent oxygen in helium mixture. The failure of variations in oxygen tension to exert any effect, so long as the partial pressure remained above the limiting value of 15–20 mm. Hg, is also visible in these series, as it is in a comparison of results obtained in air and other oxygen-nitrogen mixtures. It is evident, therefore, that the correlation between wingbeat frequency and pressure depends, in fact, upon a relationship between wingbeat frequency and gas density.

A noteworthy feature of this relationship is the relatively small magnitude of the observed effects. Even when the density of air was increased five-fold by compression, the frequency of wingbeat decreased only 16 per cent. That this effect is indeed a minor one may be judged from a comparison of the effectiveness of change in gas density with that of change in environmental temperature. The decrement that 5 atmospheres of pressure produced in wingbeat frequency can, for example, be duplicated by lowering environmental temperature only 5 or 6 degrees C.

Such small effects of variation in gas density would not be anticipated on the basis of Equation (2):

$$f^3 \propto P/m. \tag{2}$$

The mass of air moved per beat is, obviously, equal to the product of stroke volume (V_s) and gas density (ρ). Hence

$$f^3 \propto P/V_s\rho. \tag{4}$$

If the power output (P) and the stroke volume (V_s) remain constant, then frequency (f) should vary inversely as the cube root of gas density; that is, as $\rho^{-0.33}$.

The large deviation of the actual relationship from this theoretical one is amply evident in Figure 4. Equations fitted by the method of least squares to the data obtained with *D. repleta* in air at 25 degrees C. are

for densities below 1.05 gm./L.,

$$\log f = 4.1271 - 0.0460\ (\log \rho + 0.3529)\,; \tag{5}$$

for densities above 1.05 gm./L.,

$$\log f = 4.0659 - 0.1028\ (\log \rho - 0.4463). \tag{6}$$

With *D. virilis,* tested at two temperatures over the pressure range from 2 atmospheres to 200 mm. Hg, the relationships are:

at 19.3 degrees C.,

$$\log f = 4.0530 - 0.0885\ (\log \rho + 0.0623)\,; \tag{7}$$

at 25.9 degrees C.,

$$\log f = 4.1513 - 0.0726\ (\log \rho + 0.0748)\,; \tag{8}$$

and there is no evidence of any discontinuity.

In the latter respect, the true picture, we believe, is that presented by the studies with *D. virilis*. Here the samples of flies were more homogeneous, and all specimens were flown at all of the pressures included in the averages. Even so, differences were noted in the slopes of the curves given by different individuals. With *D. repleta*, where flies were selected at random from a mixed wild population, it happened by chance that more individuals whose performance yielded rate-density curves with small slopes were tested at the lower pressures, and more individuals giving greater slopes at higher pressures. Averaging these groups together has produced a composite curve with somewhat different slopes in the positive and negative pressure ranges.

With both species, the slopes for individual animals range from about -0.03 to -0.15. These differences, which occur even in stocks reared under standard conditions, do not seem to be related to age or sex, and are not understood. It is unlikely that they depend on the factors involved in the correlation demonstrated by Reed et al. (1942), who showed that the rate of wingbeat is influenced by variations in the bodily dimensions which affect the ratio between muscle volume and the area of the wings. That this should be so is evident from Equation (4) above. Power output (P) will be proportional to the product of muscle cross-section and length; stroke volume (V_s), to the wing area. Hence, for a given air density, the wingbeat frequency will be less when the ratio P/V_s is small; i.e., when the wings are large relative to the power of the muscles which move them. But variation in the slope of the rate-density relationship cannot be ascribed to differences of this sort, for alterations in the ratio, P/V_s, should yield a family of parallel curves when the logarithm of wingbeat frequency is plotted against the logarithm of density.

Accepting these differences in slope as an unexplained phenomenon, we see nevertheless that whereas theory predicts the variation of f as $\rho^{-0.33}$, the actual measurements show f varying at a rate no greater than the -0.15 power of density. Since the theoretical relationship is based upon assumed constancy of stroke volume and power output, it is clear that one or both of these assumptions must break down when frequency changes in response to alterations in atmospheric density. Each of them must therefore be subjected to further examination.

In the absence of means for direct measurement of the power output of Drosophila at densities other than normal, we have turned to the rate of oxygen consumption as an index of this factor. The oxygen uptake gives a measure of the rate at which chemical energy is liberated by the active muscles and this figure, the power input (P_i), is related to P, the power output, through a factor, e, which represents the overall efficiency of the flight process:

$$P = eP_i. \tag{9}$$

Measurements reported above (Table 9) show that P_i is essentially independent of variation in density over the range from 200 mm. Hg to 760 mm. Hg, or suffers at most a slight decrease at the lower pressure. Apparently, the rate at which the muscles are able to liberate energy is limited largely by temperature and the physiological state of the insect in respect to fatigue, so that we may with reasonable safety extrapolate our findings at reduced pressures to cover the range of positive pressures in which measurements of oxygen consumption were not feasible with our apparatus, especially since we know that substitution of oxygen for air at normal

pressure is without effect on the rate of oxygen consumption during flight. If this reasoning is accepted, we may then conclude that the power output also should be independent of density provided that varying the latter does not cause changes in efficiency.

In attempting to decide this last question, we are again hampered by lack of data, for to settle the problem would require measurements of both power output and power input at several positive and negative pressures. One might perhaps anticipate some decrement in the efficiency of the wings at reduced pressures. Should this occur, it would help to account for the failure of wingbeat frequency to rise as rapidly as predicted by Equation (4) when density is decreased; but it seems very unlikely that a several-fold increase in efficiency occurs at a pressure of 5 atmospheres, as the logical extension of this argument to the range of positive pressures would demand. Since the relationship between wingbeat frequency and density for the individual insect is continuous, without change in exponent over the entire range tested, it follows that any compensatory alteration which would account for the divergence from a line of slope − 0.33 must also be continuous. We are thus led to infer that changes in efficiency must be relatively unimportant when wingbeat frequency is altered as a function of density.

By elimination, then, we are persuaded to look upon changes in stroke volume as the most probable source of the compensation needed, and we must now inquire whether differences of the required magnitude are reasonably likely. Taking an average value of − 0.10 for the slope of the empirical rate-density relationship, we may set

$$f^3 \propto \rho^{-0.30}, \tag{10}$$

and on substitution of this value into Equation (4) we obtain, with constant power output,

$$V_s \propto \rho^{-0.70}. \tag{11}$$

On this basis, if stroke volume at normal density is taken as 1, values of about 4.4 and 0.3 would be required at 100 mm. Hg and 3860 mm. Hg respectively.

Our judgment as to whether alterations of this size are within reasonable limits will be assisted by the following analysis. Consider that the volume, V_s, swept out by the wings in each cycle is approximately the segment of a cylinder. The radius of this cylinder is the wing length, L; its height, h, is equivalent to the product of the average wing width, W, and the sine of the angle of attack, α:

$$h = W \sin \alpha. \tag{12}$$

The angle of attack is defined as the angle between the chord of the wing and the relative wind; thus the effective height of our hypothetical cylinder is the projection of the mean width of the wing on a plane perpendicular to its direction of motion relative to the oncoming column of air.

Now the volume of the segment swept out by the wings in a complete cycle, including both up and downstroke, will be related to the volume of a cylinder with the above dimensions as twice the stroke amplitude is to 360 degrees. Remembering that there are two wings, we may then summarize as follows:

$$V_s = 2\pi \times L^2 \times W \sin \alpha \times (2 \times \text{amplitude}/360°), \tag{13}$$

or, since the wing dimensions are constant for a given specimen,

$$V_s \propto \text{amplitude} \times \sin \alpha. \tag{14}$$

Thus we see that the principal variables involved in the stroke volume are the stroke amplitude and the angle of attack.

From head-on photographs of *D. repleta* in flight the amplitude at normal pressure in a plane transverse to the body axis has been measured as approximately 135 degrees with the wing tips making contact at the extreme of the upstroke. While this arc could theoretically be cut to 45 degrees to account for the required decrease in stroke volume at a pressure of 5 atmospheres, although the decrement in amplitude observed visually does not seem this large, the maximal extension possible (to a value somewhat above 180 degrees) would fall far short of supplying the 4-fold increase needed at 100 mm. Hg. For this reason it is apparent that the necessary changes in stroke volume must be effected in part through alteration in the angle of attack.

Since the stroke volume will vary with the sine of α, which for small values of the angle changes approximately as the angle itself, the hypothesis seems acceptable that the insect utilizes this mechanism in partial compensation for changes in air density. Lacking information to the contrary, we may conjecture in analogy with larger airfoils that the insect wing operates most efficiently under normal conditions with small values of α in the range from 0 degrees to 5 degrees, which are increased at densities lower than normal and reduced at higher densities. The same mechanism is familiar, of course, in the variable-pitch propellors of modern aircraft.

In summary, then, we may state that the wingbeat frequencies observed at densities other than normal are understandable only in terms of simultaneous variation in another element of the wing movement. A survey of the possibilities suggests that this must be the stroke volume, and that a part of the compensation derived from this source may be attributed to alterations in stroke amplitude, a reduction of which has been observed but not measured at higher densities. The larger share of the necessary variation in stroke volume comes apparently from small changes in the angle of attack. These have not been measured nor, since the direction of the relative wind must vary continuously as the wing sweeps through its complex path, does it seem likely that they will be. Power output and input, and the overall efficiency linking them, are probably essentially independent of variations in density.

The fact that the strain which results from alteration in the stress imposed by air resistance is distributed over several elements in the wing movement suggests analogies between the latter and other physiological functions in which homeostasis is observed. Wingbeat frequency changes less with alterations in density than if it alone were to compensate, while the deduced changes in stroke volume appear to be shared between alterations in amplitude and variations in the angle of attack. With regard to wingbeat frequency we know that it is governed largely by such physiological and environmental factors as substrate concentration (Williams, Barness and Sawyer, 1943), temperature, age and sex and we may imagine that these set a tempo of neuromuscular activity from which the organism has difficulty in departing even when confronted with major variations in other external influences. The way in which stroke amplitude and angle of attack may be regulated is unknown, but their dependence on density suggests the possibility of reflex con-

trol, mediated perhaps by campaniform receptors on the wings in response to variations in the amount of bending caused by air resistance.

Summary

Wingbeat frequency of *Drosophila repleta* Wollaston was measured stroboscopically at 25 degrees C. as a function of atmospheric pressure, over the range from 100 mm. Hg to 3860 mm. Hg in air, in nitrogen-oxygen mixtures containing either more or less oxygen than air, and in two helium-oxygen mixtures. Similar measurements were made with *D. virilis* Sturtevant at 19.3 degrees C. and 25.9 degrees C. in air over the pressure range from 100 mm. Hg to 1520 mm. Hg; and at 760 mm. Hg, 25.9 degrees C., in a mixture of 6.1 per cent oxygen in nitrogen.

The flight response was inhibited when total pressure was less than 80 to 100 mm. Hg, or when the oxygen tension was less than 15–20 mm. Hg. Increasing the partial pressure of oxygen above the value for air did not increase the rate of wingbeat.

Within the limits of experimental error, the rate was found equal at equal densities, irrespective of the medium in which it was measured. Wingbeat frequency is therefore independent of total pressure as such, and varies inversely in a logarithmic relationship with the density. The exponents measured for this relationship varied with different individuals between -0.03 and -0.15, approximately.

The helium-oxygen mixtures had a detrimental effect on the response of the insects, which was less evident at higher pressures and reversed when the specimens were returned to air.

Amputation of the halteres did not disturb the relationship between wingbeat frequency and density.

Clipping portions from the wingtips increased the frequency of wingbeat. When only one wing was clipped, the increase was less than when both were shortened by equal amounts.

Oxygen consumption of *D. virilis* was measured during flight in an oxygen atmosphere at 19.3 degrees C., at 760 mm. Hg, 400 mm. Hg and 200 mm. Hg, and was found to be relatively unaffected by variation in density.

Since wingbeat frequency varied less rapidly with changes in density than would be expected if both power output and stroke volume were to remain constant, it is reasoned that partial compensation is effected through adjustments in stroke volume. A decrease in stroke amplitude was observed at higher densities, but it appears unlikely that amplitude can increase enough at lower densities to account for the stroke volume required. Arguments are given to show that the remaining compensation needed may be furnished by alteration within reasonable limits of the angle of attack.

It is concluded that insect flight exhibits homeostatic characteristics, in that the strain which results from density change is distributed over several elements in the wing motion.

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ACTION OF ACETYLCHOLINE, CARBAMINOYL-CHOLINE (DORYL) AND ACETYL-B-METHYL-CHOLINE (MECHOLYL) ON THE HEART OF A CLADOCERAN

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This paper deals with the action of acetylcholine, carbaminoyl-choline, and acetyl-B-methyl-choline on the heart of the cladoceran *Simocephalus vetulus*. Our interest in this work is primarily centered on the comparative physiological evaluation of these chemicals with regard to their stability, potency of action, and their pharmacological effects on the heart as compared with the effects on the intestine of this animal and *Daphnia magna* (Obreshkove, 1941; Mooney and Obreshkove, 1948). A correlation is also made of the observations presented in this paper with certain facts pertaining to the role which tese drugs have been said to play in the transmission of nervous impulses.

Methods

In this study *Simocephalus vetulus* in their second instar were utilized. The method of rearing and selection of animals for the experimentation as well as the method employed in administering the drugs have been described elsewhere (Obreshkove, 1930, 1941). A single individual in each case was transferred to a micro-culture slide for treatment with the specific drug, and for examination of the activity of the heart under the microscope. After the transference of the animal to the slide, there was usually observed a slight excitation of the heart which persisted for about 15 seconds. One minute was therefore allowed to elapse before the actual readings were taken. Since the animal is seen at all times to ingest particles and fluid with which it comes in contact in the depression slide, it is assumed that the drugs employed in this work were administered orally. The rate of the heart beat was recorded on paper with penicil dots in synchronic rhythm with the heart contractions.

Acetylcholine

It was shown in the study of the effects of acetylcholine on the intestine of *Daphnia magna* and *Simocephalus vetulus* reported previously that when this drug becomes effective, it exhibits its action to the fullest extent with an abrupt, powerful, contractile wave of considerable amplitude. The vigorous peristaltic waves become further intensified with lapse of time, persist for several hours, and terminate in intestinal contracture. The effectiveness of the drug on the heart of *Simocephalus vetulus* is best expressed by the time required for the establishment of the maximum rate of contraction rather than by the time required for the production of an initial effect. In the heart, as in the intestine, there was demonstrated a graded action over a wide range of concentrations. Acetylcholine ranging in concentration from

1×10^{-2} to 1×10^{-9} was employed. In Table I are given the results obtained with 2 concentrations of the drug. It may be seen that when *Simocephalus vetulus* young are treated with acetylcholine, an increase in the rate of the heart beat is observed. Although prolonged consecutive readings were taken for each animal after the application of this and the other drugs employed, in a number of tables

TABLE I

Effect of acetylcholine 1×10^{-5} and acetylcholine 1×10^{-2} on the heart rate of Simocephalus vetulus

Normal rate	Acetylcholine 1×10^{-5}		Normal rate	Acetylcholine 1×10^{-2}	
Beat/min	Min	Beat/min	Beat/min	Min.	Max beat/min
244	2	242	274	5	314
	4	260			
	10	296*			
	15	200			
261	2	282	276	5	314
	5	270			
	10	293*			
	15	207			
277	5	286	264	6	303
	10	295			
	15	309*			
	20	278			
276	4	288	268	5	302
	10	299			
	12	303*			
	15	286			
280	5	278	270	5	318
	10	305*			
	15	194			
275	5	310	272	3	300
	10	301			
	15	315*			
	20	224			
284	5	298	274	4	304
	10	314			
	13	316*			

* Max. rate.

presented here, only the maximum heart rate was recorded. With acetylcholine 1×10^{-5} the first evidence of increased cardiac activity appears about 2 minutes after the application of the drug and a maximum rate is established in about 10 to 15 minutes. This is followed by a gradual decline in the heart beat to a subnormal rate which persists for some time. Baylor (1942) also reports a depressing effect of acetylcholine on the heart rate of *Daphnia magna* some minutes after the applica-

tion of the drug but failed to observe the acceleratory action described here. Stronger solutions of acetylcholine appreciably reduce the time required for the production of the maximum cardiac activity in *Simocephalus vetulus*. Acetylcho-

TABLE II

Effect of prostigmine 1×10^{-1} when employed alone and the effect of acetylcholine 1×10^{-5} when preceded by 2 minutes treatment with prostigmine 1×10^{-4} on the heart rate of Simocephalus vetulus

Normal rate	Prostigmine		Normal rate	Acetylcholine 1 × treatment with pr	
Beat/min	Min	Max beat/min	Beat/min	Min	Max beat/min
230	20	268	284	2	321
261	15	294	275	2	320
290	15	319	278	2	325
271	20	329	260	2	306
263	15	298	273	2	309
266	15	294	269	2	297
278	20	308	272	2	303
268	20	299	265	2	294

TABLE III

Effect of mecholyl[1] 1×10^{-5} on the heart rate of Simocephalus vetulus following atropine 1×10^{-5}

Normal rate	Atropine 1×10^{-5}		Mecholyl 1×10	
Beat/min	Min	Beat/min	Min	Beat/min.
272	5	271	2	272
			7	271
			10	269
			30	265
261	5	266	1	262
			3	265
			12	266
			30	262
264	5	263	2	260
			5	262
			10	266
			22	256
286	5	282	2	281
			5	282
			10	278
			15	285
276	5	275	2	282
			4	261
			6	280
			8	268

[1] Mecholyl—trade name for acetyl-B-methyl-choline.

line 1×10^{-2} produces the maximum effect in a period which varies from 3 to 6 minutes as compared with the 10 to 15 minutes required by acetylcholine 1×10^{-5} (Table I). When acetylcholine (1×10^{-5}) is preceded by prostigmine (1×10^{-4}) the maximum effect appeared in about 2 minu-tes (Table II) in comparison with the 10 to 15 minutes when acetylcholine 1×10^{-5} was employed alone A similar

TABLE IV

Effect of Doryl on the heart rate of Simocephalus vetulus; abolishing of this effect by atropine, and reestablishment of the Doryl effect*

Normal rate	Doryl 1×10^{-10}		Atropine 1×10^{-6}		Doryl 1×10^{-5}	
Beat/min	Min.	Maximum beat/min	Min	Beat/min	Min	Maximum beat/min
284	3	323	2 3	271 273	3	301
261	2	290	2 3	263 265	3	284
268	4	287	1 5	266 251	3	293
265	3	290	2 3	250 253	3	293
273	1	295	2 4	282 270	3	288
281	3	296	1 2	261 263	3	304
281	3	308	2 5	304 270	3	292
269	1	298	2 5	283 260	1	288
276	3	304	1 4	297 275	2	299
266	3	304	1 4	285 271	2	298

* Doryl —trade name for carbaminoyl-choline.

action was demonstrated for physostigmine in the production of an intensification of the Doryl and Mecholyl effects. The observations are in accord with those made on the intestine of this animal (Mooney and Obreshkove, 1948). Likewise when prostigmine was administered alone, it produced the same effect as acetylcholine but required on the average a longer period of time for the production of the maximum effect (Table II).

Acetyl-B-methyl-choline (mecholyl)

Acetyl-B-methyl-choline exerts a characteristic effect on the heart of *Simocephalus vetulus* in eliciting a cardiac acceleration in a manner similar to that of acetylcholine. It appears that this drug is more potent on the heart than acetylcholine as judged by the time required for the production of the maximum excitatory action when drugs of the same concentration are employed. A very clear antagonism was found to exist between acetyl-B-methyl-choline and atropine. In a series of 5 experiments a preliminary application of atropine 1×10^{-5} for 5 minutes prevented the appearance of the characteristic effect ascribed to Mecholyl even 30

TABLE V

Effect of Mecholyl 1×10^{-5} on the heart rate of Simocephalus vetulus and the abolishing of this effect by atropine 1×10^{-5}*

Normal rate	Mecholyl 1×10^{-6}		Atropine 1×10^{-5}	
Beat/min.	Min.	Max. beat/min.	Min.	Beat/min.
260	3	293	2 3	264 258
277	3	302	1 3	275 278
289	3	308	1 3	284 286
282	4	306	1 2	283 280
271	1	305	1 3	284 280
268	1	301	2 3	267 270
270	2	316	2	278

* Mecholyl—trade name for acetyl-B-methyl-choline.

minutes after the application of the latter drug (Table III). The same concentration of atropine was found effective when added after Mecholyl. These observations are similar to those reported for the intestine of this animal.

Carbaminoyl-choline (doryl)

The heart of *Simocephalus vetulus* responds even to a very weak solution of carbaminoyl-choline. A maximum rate of heart beat is produced by a low concentration of this drug (1×10^{-10}) in a comparatively short period of time. Whereas atropine was shown to abolish rapidly or prevent completely the action of acetyl-B-methyl-choline, when the doryl effects are abolished by atropine, they are quickly

reestablished after the atropine treatment is followed by carbaminoyl-choline (Table IV). Prolonged washing reduces and in time completely abolishes the effects of carbaminoyl-choline and of all the other drugs employed in this work. In abolishing the action of the drugs, the effects of washing were found to be considerably slower than those produced by atropine.

TABLE VI

Action of Doryl 1 × 10^{-5} followed by washing with distilled water on the heart rate of Simocephalus vetulus (second day young)

No.	Normal	Doryl 1 × 10^{-5}%		Distilled H_2O	
	Beats/minute	Minutes	Beats/minute	Minutes	Beats/minute
1	238	2½	289	2	281
		5	285	5	279
				10	258
				15	257
				20	250
2	256	2½	294	2	290
		3½	295	4	291
				6	289
				10	276
				15	270
3	282	2	301	2	304
		2	307	4	300
				10	287
				15	288
4	277	2	298	2	299
		3	301	4	301
				10	285
				15	272
5	269	2	296	2	294
		3½	303	4	291
				10	274
				15	270

DISCUSSION

Our endeavor to demonstrate the nerves distributed to the heart of *Simocephalus vetulus*, so important for the further clarification and analysis of the results presented here, was beset with many difficulties. It was demonstrated earlier, however, that if the intestine of *Daphnia magna* is touched with a fine glass needle at the bend of the digestive tube where the stomach enters the intestine, the heart immediately ceases to beat and after a certain period, depending on the degree of the mechanical stimulation applied, the heart escapes from inhibition (Obreshkove, 1942). The heart in this respect behaves similarly to the inhibition produced after electrical excitation of the vagus nerve in vertebrates. When *Daphnia magna* was treated with acetylcholine, prior to the production of inhibition or during the heart

inactivity, the period of cardiac inhibition was observed to be considerably shorter. It must be pointed out that whereas acetylcholine has been shown to produce cardiac excitation in some invertebrates, in others it produces inhibition. Prosser (1942) after an extensive review of the literature presents evidence to indicate that the invertebrate hearts may be grouped into three classes with respect to the action of acetylcholine and suggests that those hearts which are accelerated by the drugs are neurogenic, those which are inhibited are myogenic and those which are unaffected by acetylcholine are non-innervated.

Acetylcholine, acetyl-B-methyl-choline, and carbaminoyl-choline produce cardiac excitation in *Simocephalus vetulus*. This action of the drugs is antagonized by atropine and augmented by prostigmine and physostigmine. These and the other observations recorded in this paper are of such a nature as to suggest strongly some known facts pertaining to the role which acetylcholine and other cholinergic drugs have been said to play in the transmission of nervous impulses. The demonstration by Artemov and Mitropolitanskaja of an acetylcholine-like substance in Daphnia (1938) adds further interest to the problem.

Summary

1. Acetylcholine, carbaminoyl-choline and acetyl-B-methyl-choline produce in *Simocephalus vetulus* cardiac excitation. The heart acceleration in each case was followed by inhibition.

2. This action of the drugs is antagonized by atropine and augmented by prostigmine and physostigmine.

3. Acetylcholine exhibits a graded action for a wide range of concentrations (1×10^{-2} to 1×10^{-9}) as revealed by the time required for the production of a maximum cardiac excitation.

4. In contrast with carbaminoyl-choline, a marked antagonism was found to exist between acetyl-B-methyl-choline and atropine. When atropine is followed by carbaminoyl-choline, the Doryl effect appears in comparatively short periods of time.

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A CYTOTOXIN FROM BLEPHARISMA

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When a few paramecia were added to a concentrated suspension of *Blepharisma undulans* in a Cartesian diver, they were injured, began to rotate, and after swelling, died, although the Blepharisma remained normal and active (Giese and Zeuthen, 1949). A few individuals from a Blepharisma culture were placed with a lot of paramecia with no ill effect. An attempt was made to determine what caused the injury to Paramecium placed in a concentrated culture of Blepharisma. The results are described below.

Experimental

Cultures of Blepharisma were grown as previously described (Giese, 1938b). Practically all the other organisms were grown in lettuce infusions of the same type (0.05 per cent lettuce, buffered at pH 7.0 or 8.0), or obtained from wild cultures. *Paramecium multimicronucleatum* for division studies was grown as previously described (Giese, 1945).

In the first experiment the culture of Blepharisma was handled with great care and the animals were gently centrifuged down into the cone of a centrifuge tube. The supernatant was carefully withdrawn and after a dense suspension was available, some paramecia were added. They were in no way adversely affected. It was therefore apparent that when Blepharisma individuals are handled with care they do not liberate any substance injurious to Paramecium. The inference may be drawn that in the pipetting of the suspension of Blepharisma into the diver some individuals may have been injured. To test this possibility individuals in a dense culture of Blepharisma were fragmented by sucking the animals up into a pipette partially blocked by cotton fibers, making a "brei." In this process the animals were torn open and the fluid became pinkish.

Paramecia added to the brei reacted violently by reversed ciliary action and then quickly began moving and died. In a freshly prepared brei, the time from immersion to killing was only a few minutes. A Paramecium-brei similarly prepared was not toxic to Blepharisma nor was a Didinium-brei toxic to Paramecium. Therefore Blepharisma presents a special case worthy of further study.

Questions arise as to the nature and properties of the material liberated by Blepharisma (hereafter called the toxin without any implications other than that it is a poison of organismal origin). It is desirable to know whether the toxin is

* I am indebted to Dr. L. Garnjobst of Stanford University for a culture of Actinosphaerium, to Dr. W. H. Johnson of Wabash College for a culture of Woodruffia, and to Dr. Frederick Evans of the University of Utah for a culture of Podophrya. I am also pleased to acknowledge the skillful assistance of Mrs. Helene Leighton in the experiments on the rate of growth of Paramecium in the presence of Blepharisma and to Miss Eugenia Brandt for repetition of a number of experiments on the effects of the toxin upon a number of protozoons.

selectively injurious to Paramecium or whether it is generally toxic to organisms. Secondly, the possible function of this toxin is also of interest. Thirdly it is desirable to identify the toxin with some cellular constituent. Experiments attempting to answer these inquiries are described below.

To determine if the toxin liberated by Blepharisma is specific to Paramecium or generally injurious, a wide variety of protozoa were tried. In no case were the protozoans found to be resistant to the Blepharisma-extract, although some were more susceptible than others. *Frontonia leucas* was found to be very susceptible, and it and *Urocentrum turbo* were more susceptible than *Colpidium colpoda* placed in the same brei. The latter was more susceptible than *Paramecium multimicronucleatum* and *P. aurelia*. *Paramecium bursaria* (green). *Stylonychia curvata, Euglena gracilis, Amoeba proteus* and *Actinosphaerium eichhorni* were found to be more resistant that *P. multimicronucleatum*. Even Rotifers were observed to be affected. Not alone are infusorians injured. Blastulae and gastrulae of the sea urchin, *Stronglyocentrotus purpuratus,* were exposed to small amounts of the substance. They ceased swimming and did not recover; in a few hours they had disintegrated. The material liberated by fragmented Blepharisma individuals seems to be a rather general cellular toxin.

To determine whether the toxin were liberated in limited quantities, a succession of additions of paramecia was made and it was found that whereas the second batch was readily killed, thereafter, the time for killing increased until after many additions there appeared to be no injury. The material appeared to be adsorbed or absorbed by the paramecia and so removed from the solution.

Attempts were made to wash paramecia free of the toxin when they had shown only the first signs of injury, for example, reversed ciliary activity. In no case was the injury reversible, but became more and more pronounced until the paramecia died. Therefore, the toxin seems to become firmly attached.

To determine whether the toxin is injurious to Blepharisma itself, individuals were exposed to a freshly prepared brei. They were not affected and, in fact, they began to clean up the fragments of the corpses as could be seen by the deep red vacuoles within them. Some become giants (see Giese, 1938b, for an account of gigantism in this species). They seem unaffected by having the smaller fragments of their fellows inside them and the toxic material outside. Division was observed to occur and a healthy culture was established. Since many very minute living individuals were also observed in a culture fragmented by passage through cotton fibers, it seems likely that some of the fragments regenerate. The conclusion may be drawn that the material liberated by fragmented Blepharisma while toxic to other forms, is not toxic to itself.

Since the exudate of Blepharisma is so toxic one wonders whether it functions in preventing attack by other organisms. In that case one might expect that carnivorous protozoans would avoid attacking Blepharisma. To test this, carnivores were placed with Blepharisma. *Didinium nasutum*, a particularly voracious ciliate, which attacks Paramecium and Colpidium, avoids Blepharisma. Didinium will starve to death in the midst of a rich culture of Blepharisma but also ignores such colorless forms as Stylonychia. Another ciliate, *Woodruffia metabolica,* also attacks *Paramecium* but ignores Blepharisma as well as many other ciliates. Also the suctorian *Podophrya fixa* feeds upon Paramecium and Colpidium but starves in a culture of Blepharisma. At about the time when it appeared likely that no carni-

vores would eat Blepharisma, Actinosphaerium was tried. Not only did this heliozoan feed upon Blepharisma but it did so voraciously and individuals of the latter were not only engulfed but digested. Almost as soon as a suspension of Blepharisma was added some were caught in the extended axopodia of the heliozoan. Sometimes on struggling they succeeded in breaking loose, but more often they did not. Within a few minutes they were engulfed in the streaming protoplasm and enclosed in a vacuole which was drawn towards the body. After several hours some individuals of Actinosphaerium had as many as twelve deep red vacuoles. After several more hours they were surrounded with red fecal deposits.

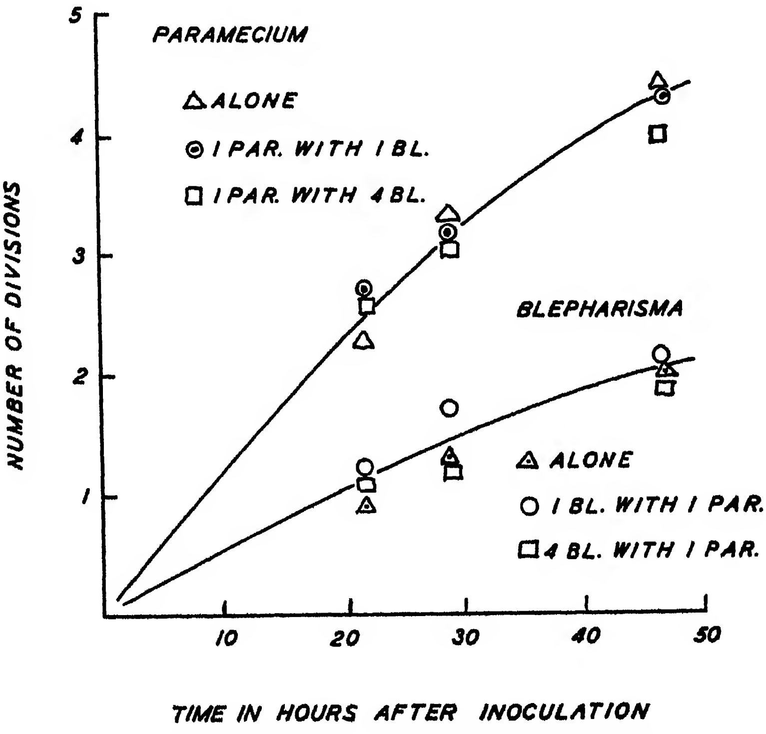

FIGURE 1. Comparison of division-rates of Paramecium in the presence and absence of Blepharisma. Three sets of eight cultures each were used for these determinations. In addition another series in which two instead of four Blepharisma were studied and still another with eight Blepharisma. All the experiments indicated the same result.

If one observes the food vacuoles within an Actinosphaerium, one will see that the contents decrease in size and become more intensely colored. As digestion proceeds the vacuoles do not seem to change hue, always appearing red. Sometimes the fluid within the vacuole turns pink. However the protoplasm of Actinosphaerium never takes on a reddish color. Actinosphaerium fed on Blepharisma continues to grow and divide. Whether division would go on at the same rate as on other food could not be determined since division occurred in such an erratic manner. The latter is probably due to the fact that Actinosphaerium is multinucleate and may

grow to a large size before dividing. The experiments with Actinosphaerium demonstrate that in spite of its toxin, Blepharisma is not necessarily protected from carnivores.

Another possible function of the toxin in Blepharisma suggests itself for testing. Perhaps the toxin excludes other species of animals when it accumulates in a culture during growth. This could be tested by growing Blepharisma together with another species in the same culture. Such experiments were performed with Paramecium and Blepharisma. A single specimen of *Paramecium multimicronucleatum* and of Blepharisma placed together in a tube of culture medium grew at about the same rate at controls grown separately. A single specimen of Paramecium placed with four individuals of Blepharisma also grew as well as the control in the absence of Blepharisma. The data are summarized in Figure 1. The conclusion may be drawn that if something is exuded from Blepharisma during growth it is insufficient to prevent paramecia from growing at least as rapidly as they would in the absence of Blepharisma.[1]

The possibility that the toxin might be the reddish pigment suggests itself since in experiments on the effects of the brei on paramecia and some of the other colorless forms it was noted that the animals became reddish after they were injured. If this were true then if the pigment were first destroyed by bleaching one might expect the brei of such animals to be innocuous. Accordingly two experiments were tried. In the first the individuals in a culture of Blepharisma were killed and disrupted by exposure to visible light (for method see Giese, 1946) and the light treatment was continued until relatively little color remained. To this material, paramecia were added and it was found that there was little if any observable effect on them. In the second set of experiments the culture of Blepharisma was first bleached by exposing it to weak light (Giese, 1938a). This was accomplished by placing it near a 6-watt daylight fluorescent lamp cooled by a fan. From the animals bleached for 24 hours a brei was made and it proved completely non-toxic to Paramecium. The material which is toxic is therefore photolabile. However the pigment might merely act as a photosensitizer to some other constituent such as a protein or fat of the cell which when affected becomes toxic. This might be answered by separating the pigment from the fats and proteins of the cell.

The pigment was next extracted with absolute alcohol (Emerson, 1935) from animals concentrated into a small red mass by centrifuging. It was then freed from particulate detritus by centrifuging and dried in a water bath and was reextracted with absolute alcohol and again dried in another dish. It was then extracted with water. Only a portion of the original pigment went into aqueous solution which was clear and reddish. From the solubility properties it would appear that the toxic substance of Blepharisma is not related to the killer substance paramecin (Sonneborn, 1948; van Wagtendonk, 1948) produced by some strains of *Paramecium aurelia*.

[1] One unexpected result of growing Paramecium and Blepharisma together is the formation of Blepharisma giants which eat Paramecium. This occurs only in cultures with *P. aurelia;* at least it was never observed in the cultures with *P. multimicronucleatum.* It is probable that the latter species is just too large to be engulfed since even when specimens of Blepharisma had become very much enlarged as a result of feeding on smaller species, they did not succeed in ingesting the larger species of Paramecium.

Specimens of Paramecium introduced into diluted aqueous pigment solution reacted much as they did to the crude material from crushed Blepharisma. They showed very strong reversed ciliary activity, then began to rotate; and as the contractile vacuoles ceased working, the animals became enlarged and died. Upon dying they became distinctly stained with a reddish tinge. While it is not certain that something toxic is not combined with the pigment, such preliminary trials as have been made using absorption column analysis indicate a single substance. The tentative hypothesis is put forth that the pigment is the toxic material. This can only be tested further by purification and study of the pigment. Such experiments are under way.

Summary

1. A brei of fragmented Blepharisma contains some substance which is quite toxic to Paramecium and a variety of other protozoans and to sea urchin larvae, but it is not toxic to Blepharisma itself.

2. Paramecia suspended in a dense culture of Blepharisma are unaffected by the mere presence of Blepharisma.

3. Blepharisma is eaten by Actinosphaerium; therefore the toxin does not protect it from attack and use as food, but it is not eaten by Woodruffia, Podophrya or Didinium.

4. In the presence of Blepharisma, paramecia grow at the same rate as they do alone, indicating that no toxin is secreted during growth.

5. Brei of Blepharisma bleached by light is not toxic to paramecia.

6. The pigment of Blepharisma extracted in alcohol and after drying re-extracted in alcohol and, after another drying, re-extracted in water is highly toxic to paramecia.

7. The tentative conclusion is drawn that the toxin is the pigment or something very closely associated with it.

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VIABILITY AND FERTILITY OF DROSOPHILA EXPOSED TO SUB-ZERO TEMPERATURES[1]

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Introduction

The dependence of the effects of x-irradiation on various physical variables has provided an effective tool bearing on the problem of chromosome breakage and gene mutation induced by such irradiation. The study of one of these variables, temperature, is obviously limited to the range which the organism can survive. This is particularly true of a large multicellular animal such as Drosophila. Since in general low temperatures do not have detrimental biological effects unless ice crystals are produced and since the freezing point of many cells is far below what would be predicted from their osmotic pressure (Luyet and Gehenio, 1940), it seemed likely that one could extend the temperature range of experiments on Drosophila most effectively by exploring the effects of temperatures lower than 0° C. The design of the experiments to be described was dictated by the conditions of the typical x-ray treatment; certain aspects of the broader problem of viability and fertility under these conditions have therefore been emphasized, others ignored.

Materials and Methods

Equipment

Treatment was made in a specially designed cold temperature chamber, consisting of an insulated box of about two cubic foot capacity cooled by coils from a ⅓-horsepower refrigeration unit and heated simply by a hundred watt lamp. A partition divided the chamber into two sections, the upper of which was used for treatment, while the lower contained the heating and cooling units. A 1/70 horsepower blower at one end of the partition forced the air from the lower into the upper chamber; an opening over the refrigerator coils at the other end provided for the free circulation of the air. A thermostat was placed in the blower air blast from the lower section into the upper one. Thus a heating-cooling cycle was completed about every half minute. Thermocouple measurements showed that the air in the upper chamber varied during the cycles not more than 1½° C. on either side of the average temperature measured with a standard mercury thermometer. In those cases where the temperature variation was to be minimized the material was placed in a 15 cubic inch cardboard box which could be closed after the desired temperature had been reached in the chamber. The variations in the cardboard box, again meas-

[1] This work was done during the course of investigations supported by a grant of the Rockefeller Foundation to the Department of Zoology, University of Missouri.

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ured with a thermocouple, amounted to only ten per cent of those in the surrounding air blast. Cellophane windows were provided in both the removable cork top of the chamber and in the small box to facilitate checking the material during treatment and to minimize the absorption of X-rays during irradiation.

Rate of change of internal temperature

Thermocouple measurements inside the thorax of the fly subjected to an air blast at − 8° C. indicate that the internal temperature of the fly drops at an initial rate of about 1.6 C. per second, the rate becoming less as the gradient between the external and internal temperatures decreases, and that the fly and air temperatures are the same in from two to three minutes. The authors are indebted to Professor A. C. Fabergé who constructed by intricate plating techniques a very fine thermocouple for the purpose of making these measurements.

Genetic methods

The choice of stocks used in this work was determined by the plan of concurrent irradiation experiments; therefore the highly inbred Canton-S strain of *Drosophila melanogaster* was used. In fertility tests, these flies were provided with mates from the "Muller-5" strain which is now widely used in tests of irradiation effects in this species. The individuals to be treated were placed in size 00 gelatin capsules with holes punctured in both ends for rapid ventilation. Usually twenty flies at a time were placed in one capsule; numbers presented in the viability experiments therefore occur in approximate multiples of twenty which represent grouping of individual runs. Tests of fertility were made by placing single treated individuals with appropriate mates in 8 dram shell vials containing standard cornmeal-molasses-agar Drosophila culture medium.

Results

General behavior at low temperatures

As the internal temperature of the fly drops, it becomes sluggish and at + 3° C. all movement stops. A normal posture is then maintained regardless of the extent of the subsequent decrease in temperature. Lethality manifests itself during the period of recovery from the cold treatment. If the treatment is too severe, the fly assumes a posture characteristic of death by overetherization, i.e. the wings are held parallel in an upwards position. In some instances sub-lethal temperatures have impaired the locomotor control of the flies. Such individuals, after removal to room temperature, remain motionless or make feeble and uncoordinated attempts to walk. One such ataxic individual remained alive for 3 days; but for the purpose of this work such cases will be included with the deaths.

Effects of pretreatments

In the earliest series it became obvious that Drosophila under the influence of ether were particularly sensitive to the cold shocks. In the experiments to be described, the individuals were always allowed to recover completely from etherization before treatment. The high sensitivity under these conditions may account for the

failure of some workers (see the discussion) to use temperatures below 0° C. Other techniques were tried to increase the resistance to cold without effect; these include pretreatment with $CaSO_4$ desiccant, with temperatures less severe than the final one (1° C. for 20 minutes before exposing the fly to − 10° C.), and with a sudden exposure of the animals to a temperature lower (− 20° C.) than that of treatment.

Viability of Drosophila at low temperatures

At 0° C., Drosophila males can survive for about 24 hours; since they die in about the same time in isolated capsules at room temperature, no additional work

TABLE I

Mortality of Drosophila melanogaster males treated for various durations of time at 4 subzero temperatures. A = duration of treatment in minutes; B = number of males treated; C = number killed by treatment; D = percentage of mortality.

−5° C.														
A	10	20	30	40	50	60	70	80	90	100	110	120		
B	20	40	60	40	20	60	80	82	80	40	20	40		
C	1	0	1	1	0	3	5	41	54	2	11	30		
D	5	0	1.6	2.5	0	5	6.3	50	68	5	55	75		
−10° C.														
A	10	20	21	22	23	24	25	26	27	28	29	30	40	50
B	60	178	60	60	20	60	20	60	20	60	20	237	80	20
C	6	27	1	1	2	22	20	59	20	60	20	228	80	20
D	10	15.2	1.6	1.6	10	36.7	100	98.4	100	100	100	96.2	100	100
−15° C.														
A	1	2	3	4	5	6	7	8	9	10	11	12	13	13
B	20	20	20	20	40	20	40	40	20	20	20	20	20	180
C	1	7	9	4	16	3	15	23	16	14	17	13	15	180
D	5	35	45	20	40	15	38	58	80	70	85	65	75	100
−20° C.														
A	.5	1	1.5	2										
B	20	20	20	20										
C	2	13	16	20										
D	10	65	80	100										

has been done in this range. The percentage of mortality for various durations of exposure at − 5° C., − 10° C., − 15° C. and − 20° C. are given in Table I. At − 5° C., two hours duration does not kill 100 per cent of the flies; at − 10° C., 25 minutes treatment is completely lethal; at − 15° C., 14 minutes is lethal and at − 20° C., 2 minutes. The thermocouple measurements previously referred to suggest that in all series except the last, the time required for the flies to reach the temperature of the chamber is insignificant; in the last, however, some temperature between − 15° C. and − 20° C. is lethal per se without respect to time duration. One set at − 10° C. run at 2 minute intervals from 14 to 32 minutes, with parallel sets of males and females showed identical sensitivities of the two sexes.

The sterilization of fertilized females

Preliminary tests of 32 females subjected to − 10° C. for one-half hour and subsequently mated had shown their fertility to be unimpaired. On the other hand, about 200 fertilized females exposed to − 5° for 56 minutes during an irradiation experiment proved to be sterile. Since, in the latter case, mature sperm as well as ova were subjected to the treatment, it appeared likely that the sterility was caused by an inactivation of the sperm stored in the female, or "desemination" (Muller, 1944). A number of different kinds of tests were made to determine whether the adverse effect of the low temperatures was on the ova or sperm carried by the fertilized females. In these runs, Canton-S females were placed in quarter pint milk bottles with Canton-S males for three days or longer in order to insure the insemination of most of them, the proportion fertilized being determined by a test of a sample of them made as a control. After treatment, they were placed individually in shell vials with food, and their offspring counted nineteen days later, unless otherwise noted.

TABLE II

Fertility of fertilized Drosophila females exposed to −5° C. and −10° C. for varying durations of time

Duration	Females treated	Females fertile	Offspring per fertile female	Females producing only one offspring
Temperature = −5° C.				
0	25	24	79	0
15	50	1	88	11
30	50	1	88	10
45	50	2	43	13*
60	50	1	100	8
75	50	0	—	8
90	50	0	—	6
Temperature = −10° C.				
0	25	24	92	0
5	50	0	—	12
10	36	0	—	4
15	50	0	—	6
20	50	0	—	5

* Includes one female which gave 2 offspring and another which gave 4.

Out of 50 fertilized females exposed to 0° C. for 6 hours, 7 were sterile; the untreated control showed 4 sterile out of 50. This treatment is ineffective in sterilization. A larger series was subjected to − 5° C. for periods from 0 to 90 minutes in 15 minute intervals and to − 10° C. for periods from 0 to 20 minutes in 5 minute intervals. The 0 minute series in each case represent the controls. The results are shown in Table II. It is obvious that − 5° C. does sterilize the females since only 5 out of 300 treated were fertile, whereas 24 out of 25 in the controls were fertile. The five cases of fertility after treatment at − 5° C. with durations from 15 to 60 minutes were cases of complete fertility. When the exposures were longer than 60 minutes, no completely fertile females were found. Likewise exposures to − 10° C. sharply decrease the percentage of fertile females. The

sporadic production of single, or very few, offspring by treated females noted in Table II was probably overlooked in the earlier runs.

In order to determine whether there is any effect of the cold shocks in the germ line of the female, which would account for the above results, a series was run in which fertilized Canton-S females were mated, after exposure to a sterilizing dose, to Muller-5 males. In this way it is possible to differentiate between sperm stored in the female at the time of treatment which would produce round-eyed females progeny, and the sperm introduced by the Muller-5 males after treatment, which would give narrow-eyed female offspring. In the event of an effect on the germ line of the female, no offspring of either type would be anticipated. Progeny counts were made 13 days after treatment; this short egg-laying time should make more obvious any temporary sterilization of the female which a longer egg-laying period might obscure. The results are shown in Table III. The single female which produced after treatment offspring of the first insemination yielded only one, like the sporadic cases described above. The fertility of females treated and subsequently mated is, in this run, higher than that of the untreated females not mated afterwards. This difference may be due to an increased viability of offspring of the second mat-

TABLE III

The productivity of fertilized females exposed to −10° C. for 20 minutes and subsequently mated to Muller-5 males compared with that of females so treated but not mated subsequently and with untreated and unmated females

	Untreated not mated	Treated unmated	Treated mated
Total ♀♀ treated	23	11	79
♀♀ producing offspring of first insemination	11	0	1
♀♀ producing offspring of second insemination	0	0	17
Average no. of ♀♀ offspring per fertile female	18.6	0	33.5

ing. However, the essential point is that the germ cells of the female are apparently not affected by the treatment and that such sterilization as does occur must be attributed to the killing of sperm stored in the female.

Dissections of deseminated females and their untreated sisters as controls revealed no motile sperm in the ventral receptacles or spermathecae of the former, although there was an abundance of motile sperm in both these organs of the controls. In addition, the quantity of sperm (immotile) in the ventral receptacles of the deseminated females was much smaller than that in the controls, in most cases the receptacles appearing completely empty as if a contraction had expelled the sperm. The natural striations of the chitinous spermathecal wall prevented any comparisons of quantity in the two sets, although in a few cases where a spermatheca of a treated female had been broken by pressure, sperm appeared in approximately normal quantity; they were, however, immotile.

The sporadic occurrence of one or two offspring among deseminated females may have one of two explanations. Either the treatment is not inactivating all the stored sperm, or those few offspring result from eggs already fertilized, or in the process of fertilization, at the time of treatment. These alternatives have been dif-

ferentiated in two ways. First if exposure to a deseminating dose kills all but small fraction of the stored sperm, then the application of two such doses should be effective in decreasing their incidence even more. Fifty fertilized females, subjected to two sterilizing doses of $-10°$ for 15 minutes separated by an interval of 2 hours at room temperature, produced 8 offspring, each from one treated female. Twenty-five untreated controls produced an average of 109.7 offspring in 24 fertile vials. This frequency of sporadics is of the same order of magnitude as that in the previous single shock treatments.

On the other hand, if the sporadic cases are to be accounted for by the presence of fertilized eggs in the oviduct of the female at the same time of treatment, then, since those eggs are laid first, the sporadic individuals should come primarily from the first eggs deposited. Once again 50 Canton-S females, presumably fertile, were treated with $-10°$ for 15 minutes and transferred to new culture bottles on 4 successive days. The eggs laid on the first day included 14 sporadic cases; those on the second, third and fourth, none. A similar run, interrupted after the second day, gave 8 sporadic cases in the first day, none on the second. The controls in both the above cases were highly fertile. It seems reasonable to conclude, then, that these occasional single progeny appearing after the cold treatments result from eggs which had been fertilized before the time of treatment.

Effects on fertility of the male

In marked contrast to the pronounced lethality of cold shocks on sperm stored in female Drosophila, spermatozoa in the males are more resistant to changes in temperature, although here, too, there appears to be some lethality. Thirty-eight males exposed to $-10°$ C. for twenty minutes were all fertile. Their offspring appeared in the customary ten day period, which contradicts the possibility that the mature sperm were killed and that sperm differentiating after treatment were used.

TABLE IV

Sterility and productivity of Drosophila males exposed to low temperatures with and without 3600 r. of x-rays during a 56 min. interval

	Irradiated			Unirradiated	
	25° C.	0.5° C.	−5° C.	0.5° C.	−5° C
Total treated ♂♂	65	102	115	100	100
Total fertile ♂♂	60	48	11	92	50
% fertile ♂♂	92.4	47.1	9.6	92	50
Number of female offspring/fertile male	30.1	11.8	9.4	22.4	22.4

Likewise 74 out of 158 (equals 47 per cent) of males treated with $-5°$ C. for one hour were fertile whereas a smaller untreated control series showed that 15 out of 19, or 78 per cent, were fertile. In all these cases, those males which produced any offspring at all produced the normal number. Comparable time-temperature series on fertilized females (see above) were almost 100 per cent effective in killing sperm. When males are dissected after treatment with an exposure that kills all the sperm

stored in females, there appears to be no mortality of sperm in the testes or seminal vesicle.

In agreement with the observations of others (Medvedev, 1935; Mickey, 1939) that irradiation at low temperatures decreases the fertility of males to an extent greater than that anticipated on a single additive effect basis, the data in Table IV show the fertility and productivity of males (mated singly to 2 Muller-5 females in shell vials) after exposure to 0.5° C. and − 5° C., with and without a dose of 3600 r during a 56 minute treatment. In the unirradiated series, the percentage of males completely sterilized increases with decreasing temperature, but the number of F_1 female progeny (the males not being counted for technical reasons) from the fertile males is essentially normal under the conditions of the experiment. However, with irradiation not only does the percentage of fertile males drop more rapidly, but the number of female progeny of the fertile males is between a half and a third normal. This is apparently related in part, at least, to the greater production of chromosomal aberrations during irradiation, at the lower temperatures, which will be discussed in more detail elsewhere.

Discussion

Applicability of temperatures below 0° C.

From the results of the experiments described above, it is clear that Drosophila will tolerate somewhat lower temperatures than previous workers have used. Thus, there are a number of accounts in the literature of the use of low temperatures in the range of + 3° C. to + 15° C.; in a few cases 0° C. has been reached (Medvedev, 1935; Papalaschwili, 1935; Mickey, 1939; King, 1947) and there is one instance (Kerkis, 1939) where Drosophila has been subjected to a temperature of − 6° C. It seems clear that temperatures below 0° C. are generally applicable provided that care is taken to insure complete recovery from etherization before, and adequate ventilation during treatment. This may permit a decisive test of the hypothesis that the genetic effects of x-irradiation are the immediate result of ionization, since this hypothesis predicts that the results should be temperature independent.

Desemination of Drosophila females

In many types of experiments with Drosophila, one of the most burdensome chores is the collection of virgin females. The observation that sperm stored in a female may be killed by the application of low temperatures, without affecting the fertility of the treated females in subsequent matings provides an effective tool in Drosophila work. Briefly summarized the procedure adopted for this treatment is the following: From 50 to 100 etherized females are placed in one size 00 gelatin capsule which is ventilated by pin holes at both ends. After an hour or two, during which time the flies recover completely from the etherization, the capsules are placed in a cold air blast of − 10° C. for 10 minutes or of − 5° C. for 90 minutes. Upon removal from the low temperature, they may be mated immediately if their sporadic progeny are distinguishable genetically from those of the post-treatment mating, otherwise they should be kept in a culture bottle for a day before mating to allow them to deposit the few fertilized eggs unaffected by the treatment.

Summary

1. At − 5° C. about 50 per cent mortality of Drosophila melanogaster is reached after two hours; at − 10° C. a 20 minute exposure kills very few whereas a 25 minute exposure is almost completely lethal; at − 15° C. about 50 per cent survive exposures less than 10 minutes long whereas an exposure of 13 minutes or longer is completely lethal; and at − 20° C. all individuals are killed within a few minutes.

2. Cold shocks of air at − 5° C. for 75 to 90 minutes and at − 10° C. for 5 to 20 minutes are lethal to sperm stored in adult females although such treatment has no effect on the subsequent fertility of such females. Males are not sterilized to any great extent by such exposures.

Acknowledgments

The authors wish to express their thanks to their colleagues in the Departments of Zoology and Genetics of the University of Missouri, whose active interest made these observations possible, and in particular to Professor D. Mazia who contributed many helpful suggestions as well as necessary equipment during the course of the work.

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STUDIES ON MARINE BRYOZOA. IV. NOLELLA BLAKEI N. SP.

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Introduction

During the summer of 1946 while growing some *Perophora viridis*, a Protochordate, for use of students in the Marine Biological Laboratory (M. B. L.) invertebrate zoology course, the writer noted some small delicate Nolella colonies growing in the same culture dishes. Identification of the bryozoan species was difficult for two reasons: (1) because existing descriptions of various species of Arachnidium, Arachnoidea, and Cylindroecium or Nolella in all stages of their development are not as extensive as one might hope for and (2) the present specimens were studied in the living, growing state for only eight days, from August 25 to September 2, 1946; hence, only a few colonies could be observed and these mostly in the young, developing stage. Because of the desire to report the form so subsequent workers or collectors could watch for it and study it more fully it was deemed advisable to publish the following data on the species.

Collection and Growth Data

On August 14, 1946, the Marine Biological Laboratory supply department collected a quantity of *Perophora viridis* from Lagoon Pond, Martha's Vineyard, Massachusetts. The Perophora was conspicuously overgrown with *Aeverrillia armata* and hydroids. On August 16, some Aeverrillia sprigs and the greenest Perophora stolons and buds were selected and cut into 10 to 15 mm. lengths for culturing (as in Figs. 1, 2). The watch glasses with their taped colony fragments were immersed upside down in racks (Fig. 2) in large laboratory aquaria into which natural sea water was piped from a near-by bay. In time the Aeverrillia and Perophora fragments developed colonies with stolons radiating in several directions over the bottom of the glass (Fig. 1). These watch glasses were studied daily under the microscope. On August 25 Nolella stolons and bases were discovered in ten watch glasses. These were watched daily till September 2, when observations had to terminate. The specimens were identified as one of the Ctenostomata, family Nolellidae, genus Nolella, new species. It was finally named *Nolella blakei* in honor of a most esteemed professor and kindly adviser, Dr. Irving Hill Blake of the University of Nebraska.

Morphology of Nolella Blakei n. sp.

The young zoarium is soft, transparent, and inconspicuous. It consists of thread-like "stolons" and basally adherent, upright columnar zoids whose tips are squared. Very young zoids arise as squared peristomes from flattened, enlarged bases which have temporarily serrated borders.

"Stolons"

Annandale (1907, page 199), Harmer (1915, page 43), Silén (1942, page 6) and others questioned the suitability of the term "stolon" for the long creeping processes connecting the zoids of some Ctenostomes because these processes are really extensions of the zoid bases and are sometimes not closed off by a septum from the main body cavity of the zoid. See Figures 7, 12, 13, and 19 (W) for such stolons in young *Nolella blakei*. Some of the young *Nolella blakei* stolons have septa at their origin, others do not. The younger the colonies the more apt are they not to have yet formed septa. Whether there are some non-septate "stolons" in old *Nolella blakei* individuals is not known because the older zoids (Figs. 15, 17, 18) were torn from the taped Perophora so complete stolons were not generally obtained. The following description of *Nolella blakei* "stolons" is therefore based

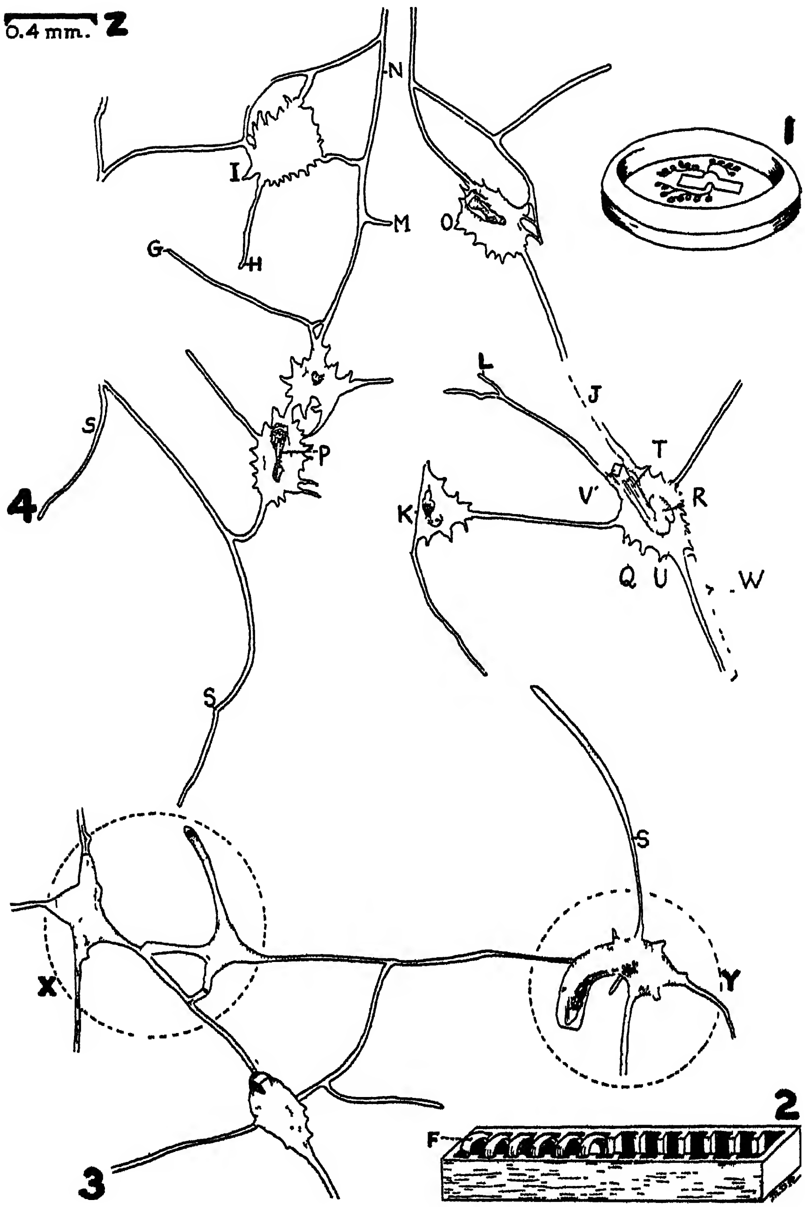
TABLE I

Measurements of Nolella blakei n. sp.

	Maximum	Minimum	Average	No. of readings
Tentacle number	12	8	10	13
"Stolons"				
Length	2.296 mm.	0.155 mm.	0.767 mm.	35
Width	.046 mm.	.015 mm.	.029 mm.	33
Upright, retracted zoid:				
Length of vertical part, exclusive of proximal "stolon"	.899 mm.	.697 mm.	.775 mm.	3
Width	.093 mm.	.076 mm.	.049 mm.	3
Basal enlargement (exclusive of stolons):				
Length	.472 mm.	.328 mm.	.397 mm.	20
Width	.341 mm.	.205 mm.	.268 mm.	20
Number of small pointed extensions, exclusive of stolons, from the basal enlargement border, in young colonies	17	4	10	19

largely on conditions in young colonies. The "stolons" are thin-walled, slender (0.015 to 0.046 mm. in diameter), anastomosing, short or long (0.155 to 2.296 mm.) and adherent along their entire length. Three to five "stolons" originate from the zoid base (Fig. 5). They are generally separated by a septum at their proximal end or point of origin from the distal end of the basal enlargement (Figs. 7, 13). "Stolons" may grow into each other and establish new connections with other zooecia (see the solid black lines in Fig. 5) in a relatively short time (two days). Zoids arise as buds from the "stolons" (Fig. 10, A). The basal enlargement itself appears to have been formed at the distal end of a "stolon" from which it was not separated, at least in young colonies. . Additional data on "stolons" and other parts are given in Table I.

PLATE I



EXPLANATION OF PLATES

All figures except 1, 2, 6 and 13 were drawn with the camera lucida. All but Figures 1 and 2 are of the new species *Nolella blakei.* Figures 3, 7, 10, 11, 12 and 14 were drawn from colonies growing in one watch glass; Figures 4, 5, 8, 9, 16 from another and Figures 15, 17, 18 from colonies in a third watch glass.

Basal enlargement

The flattened basal enlargement of *Nolella blakei* is delicate, soft, transparent, and irregular in outline. It sometimes tends toward a rough diamond shape (Figs. 7, 8, 19). It is conspicuous in young colonies having a crenulate or serrate border in young zoids but is not noticeable in the few available older zoids (Figs 15, 17,

TABLE II

Comparison of tentacle numbers in related species

Tentacle number	Species	Primary or secondary reference sources
12–16	*Arachnidium fibrosum*	Marcus, 1938, p. 51; 1941, p. 27
26–30	*Arachnidium irregulare*	Harmer, 1915, p. 49
16	**Arachnidium ray-lankesteri*	Rousselet, 1907, p. 255
18–20	*Arachnidium simplex*	Hincks, 1880b, p. 284
16	*Arachnoidea evelinae*	Marcus, 1937, pp. 130–131
16+	*Arachnoidea protecta*	Harmer, 1915, p. 50
about 18–20	***Cylindroecium dilatatum*	Hincks, 1880a, p. 536
no data	*Cylindroecium horridum*	O'Donoghue, 1926, p. 61
about 10	*Cylindroecium pusillum*	Hincks, 1880a, p. 537
no data	*Cylindroecium repens*	O'Donoghue, 1923, p. 192
no data	*Cylindroecium spinifera*	O'Donoghue, 1924, p. 59
about 10	*Nolella alta*	Marcus, 1938, p. 55
16–20	*Nolella annectens*	Harmer, 1915, p. 59
8–12	*Nolella blakei*	present study
18–22	*Nolella gigantea*	Marcus, 1937, p. 132
about 18	*Nolella papuensis*	Harmer, 1915, p. 55

* Now Arachnoidea (Harmer, 1915, p. 51).
** Now Nolella (Marcus, 1938, pp 53–55).

18) because the growing zoid cylinder gradually incorporates it. From its sides extend outward five to seventeen serrate processes (Figs. 8Q, 16) which adhere to the substratum and are apparently only of a temporary nature. In time they become obliterated by the enlarging zoid. In *Nolella blakei* these cuticular projections are

PLATE I

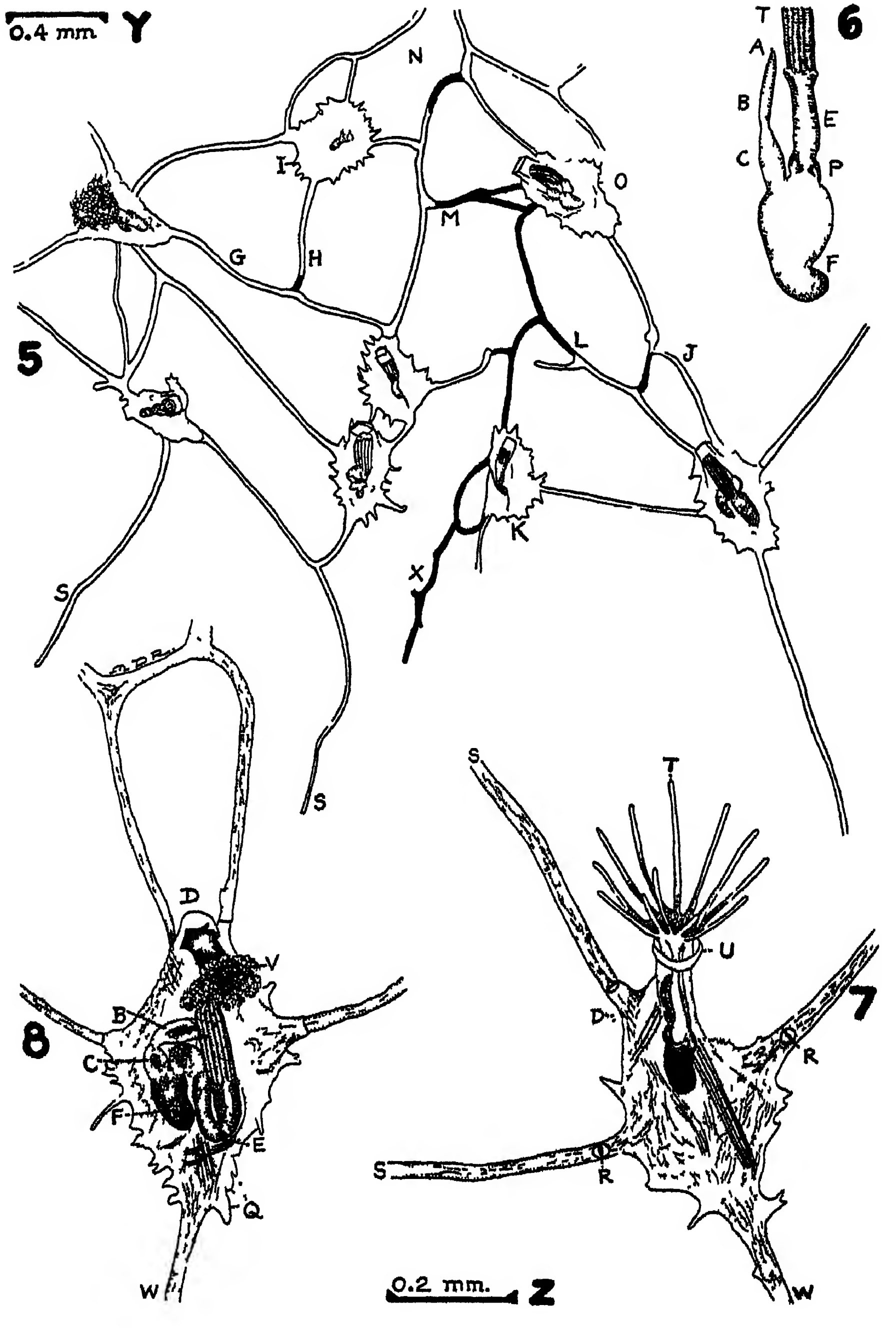
FIGURE 1. Diagram of a Perophora colony growing in a Syracuse watch glass from a fragment which had been fastened down with waterproof adhesive tape about one or two weeks before. Aeverrillia and Nolella grew under similar conditions along with the Perophora.

FIGURE 2. An open wooden rack containing several watch glasses (F) for culturing bryozoa and Perophora.

FIGURE 3. Part of very young *N. blakei* colony drawn on IX-1-1946. Circle X encloses two basal enlargements which as yet don't have a visible polypide and which are enlarged in Figure 10. Circle Y zoid is enlarged in Figure 14. Drawn to Scale Z. (S) Stolons.

FIGURE 4. Another young colony. The parts are labelled for comparison with Figure 5: (Q) basal serrations; (P) polypide; (R) gut; (T) tentacles; (V) squared peristome rim; (W) proximal extension of Zoid U and classed as one of its "stolons" (S). A transverse septum is absent from it for a considerable distance. Stolon J was damaged along the dotted area. Letters G, H, J, L, M, N all represent particular stolons in which changes occurred in the two days which elapsed between conditions depicted in Figures 4 and 5. Individuals I, K, O and U show a developmental sequence. The youngest (I) is a sac without a polypide (P). Drawn to Scale Z.

PLATE II



confined to the edge of the young basal enlargement and were not present over its upper surface nor along the upright cylinder, consequently differing in spination from *Arachnidium fibrosum*, *Cylindroecium* (Nolella) *spinifera*, *C. horridum* (*N. horrida*) and *Nolella sawayai*.

Upright columnar zoid

The peristome rises upward from the basal enlargement, lengthening into a tall cylinder in time (Fig. 15). In retracted *Nolella blakei* zoids the peristomeal orifice is squared for a short distance (Figs. 12, 15, 16) In partly or fully extended zoids this character is not particularly noticeable (Figs. 7, 8, 11, 14, 18). This condition seems to obtain for *N. papuensis* also. In *N annectens* the squaring appears to be of greater extent along the peristome than in *N. blakei*. The squared orifice distinguishes the genus Arachnoidea from Arachnidium Arachnoidea has it while Arachnidium has a rounded peristomeal orifice. The main differences between *Nolella blakei* and the Arachnoidea species are in its taller zoids and smaller tentacle number (see Table II).

As *Nolella blakei* matures its zoids lengthen greatly vertically until they resemble slender, soft-walled columns. They are flexible and can twist about slightly as Figures 11, 14, 15, 17 and 18 show.

Polypide

The polypide consists of the tentacular crown (8 to 12 tentacles), the digestive tract and associated musculature.

The digestive tract terminology is in a nice state of confusion Dr. Silén (1944) attempted to bring some order out of the chaos.

The digestive tract of *Nolella blakei* consists of the mouth, pharynx, esophagus, proventriculus (or gizzard?), stomach, intestine, rectum, and anus. The pharynx and esophagus are gray in color. The esophagus is exceedingly long in tall zoids (Figs. 15, 18). Whether the next part of the gut is a proventriculus or a gizzard could not be stated with certainty in the present material In young zoids it was the same yellow color as the stomach. In other zoids it was yellower and thicker (a band). In still older zoids it seemed to have faintly defined teeth. Harmer and

PLATE II

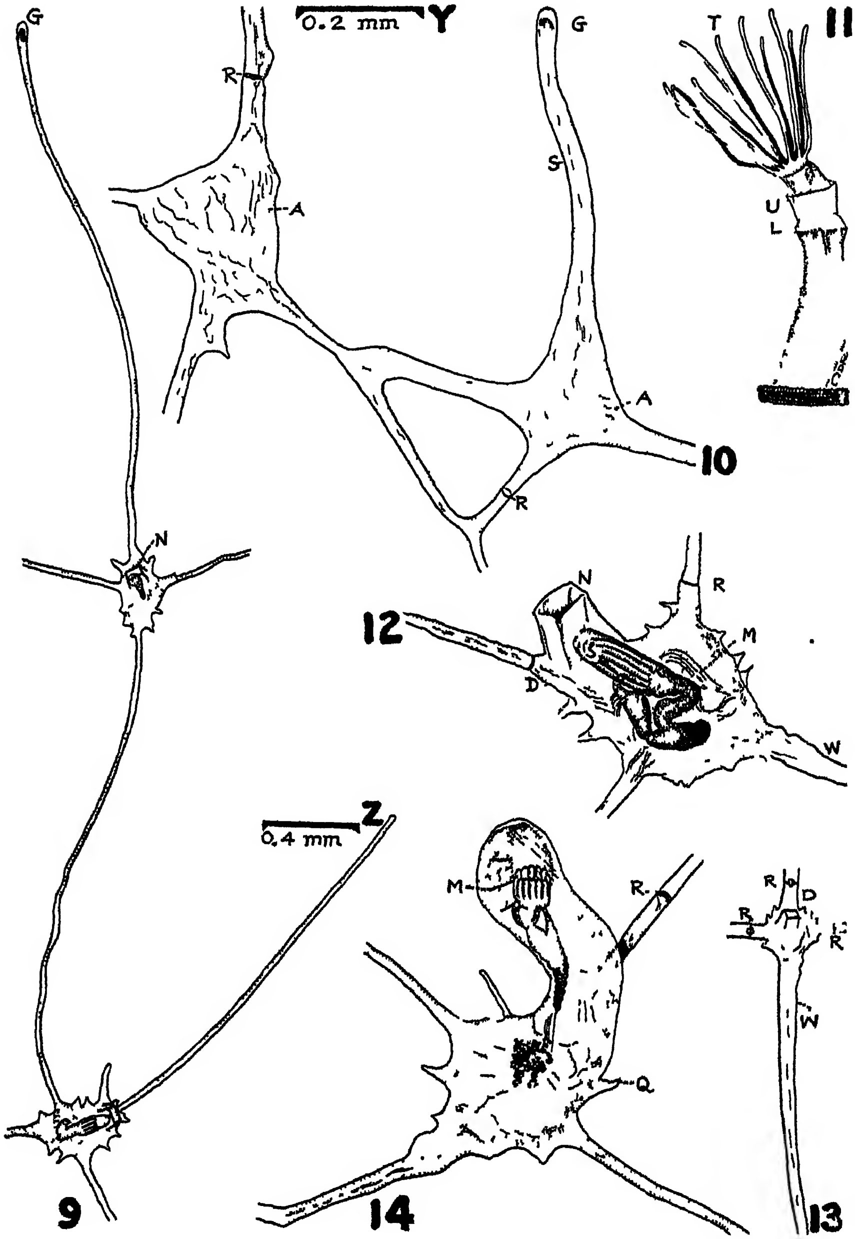
FIGURE 5. The colony of Figure 4 but drawn two days later, showing in solid black the growth and anastomoses of stolons (H, J, L, M, S, X) during that interval. The zoids (I, K, O) also have grown. Stolon N has changed its connections and the dotted part has degenerated. Drawn to Scale Y.

FIGURE 6. Diagram of polypide parts: (A) anus; (B) rectum; (C) intestine; (E) esophagus; (F) stomach or caecum; (P) gizzard? or proventriculus? and (T) tentacles.

FIGURE 7. A young zoid with 12 tentacles (T). Others in the same colony had 11. Other labels are: (D) distal part of zoid; (R) septa at the origin of the stolons; (S) stolon; (U) collar; (W) proximal extension of the zoid, one of the so-called stolons. Four stolons lead into the serrate base. Drawn to Scale Z.

FIGURE 8. A retracted zoid with 5 stolons at its base. The two distal (D) stolons anastomose. A clump of debris (V) obscures peristome area. Septa separate all stolons except the proximal one (W) from the expanded zoid base. Other labels are: (B) rectum; (C) intestine; (E) esophagus; (F) stomach; (Q) basal serrations. Drawn to Scale Z.

PLATE III



others state however that a true gizzard is lacking in this genus. The caecum or stomach is typical of various bryozoa. The intestine is narrow. The rectum is narrow and very long in tall zoids. When the polypide retracts, the gut is withdrawn into the lower part of the body cavity in a rather twisted or folded fashion (Figs. 8, 15). When the polypide begins to emerge from the vestibule the short thin membranous collar (Fig 18U) precedes the tentacular crown. Upon emergence and expansion of tentacles the collar is some distance below the tentacles (Fig 7U). It is so transparent that it is easily overlooked. With the extrusion of the tentacular crown the pull on the gut is such that it straightens out the coils and twists of the tract.

Discussion

Nolella blakei appears to be an intermediate form between the Arachnidium, Arachnoidea, and Nolella genera Its youngest zoids resemble the first two genera. Its mature zoids are definitely Nolella. The young *Nolella blakei* colonies resemble *Arachnoidea evelinae*, *A. protecta* and *A. ray-lankesteri* closely in the following respects All four species have similar "stolons," squared peristome, and serrate basal enlargement, but *Nolella blakei* has fewer tentacles, and its short peristome elongates eventually into a long vertical zoid whose basal crenulations disappear with age Other (minor) differences between *Nolella blakei* and the Arachnoidea species concern the vertical or linear extent of the peristomeal squaring and the proportionate size and diameter of peristome as compared to the basal enlargement. Also, *Nolella blakei* differs from *A. protecta* (Harmer, 1915, Plate III, Figs. 9, 10) in the much larger size of the latter's setigerous collar.

Nolella blakei resembles *Arachnidium fibrosum* (Marcus, 1938, Plate XII, Fig. 29A) in type of stolons and crenulated basal border. It differs from *A. fibrosum* in smaller tentacle number and in the absence of numerous bristle-like encrusted cuticular outgrowths originating from the peristome and basal enlargement's upper surface. Moreover, Arachnidium has a rounded peristomeal orifice rather than a squared one.

Plate III

Figure 9. An especially long young branch showing a transparent growing tip (G) and two young zoids with squared peristome (N). All septa have not yet formed. Drawn to Scale Z.

Figure 10. Eight stolons (S) and two zoid anlagen (A). This represents Circle X of Figure 3. Drawn to Scale Y.

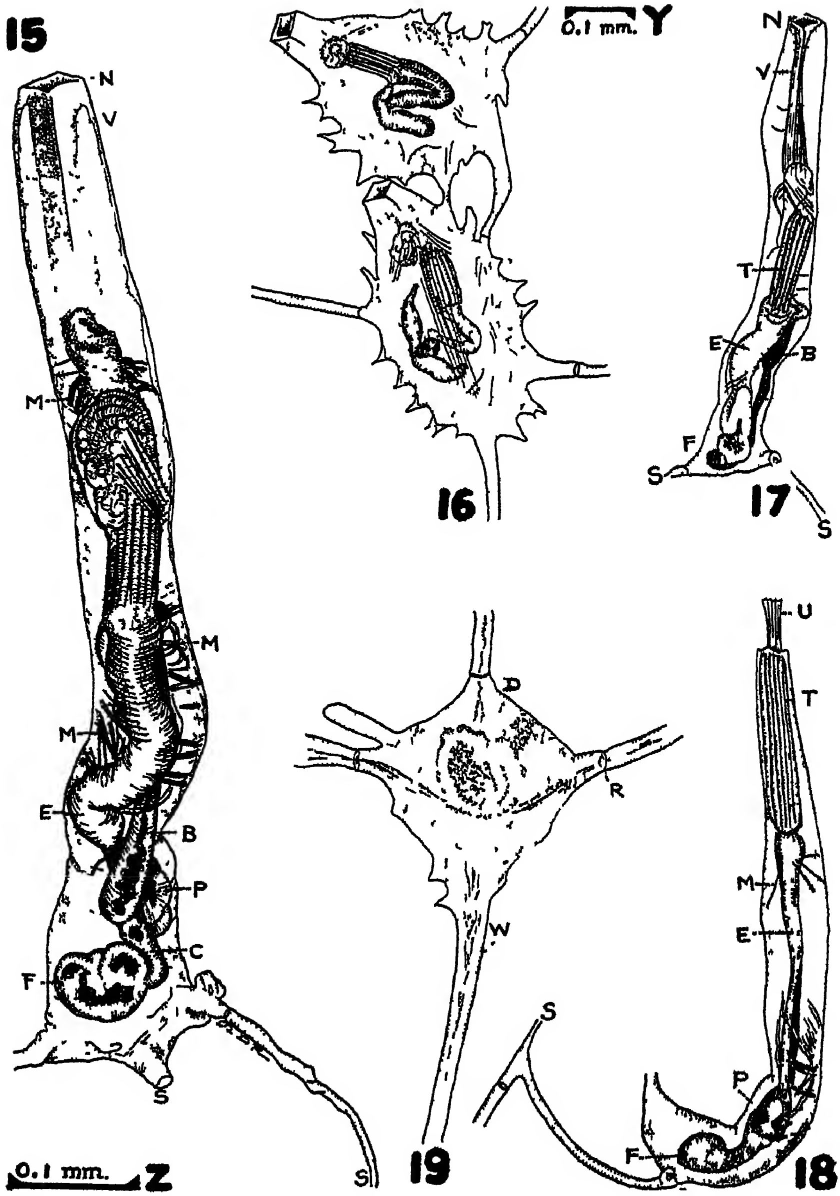
Figure 11. Upper part of a zoid with extended polypide, showing 11 tentacles (T), transparent membranous collar (U) and a slightly debris-covered peristome (L). Drawn to Scale Y.

Figure 12. Young zoid with squared peristomeal orifice (N) and retracted polypide. Other structures: (D) distal part of zoid; (M) muscles; (R) septum and (W) proximal extension of zoid. Drawn to Scale Y.

Figure 13. Diagram showing relations of stolons and septa (R), with respect to the distal (D) and proximal (W) parts of a zoid, the flattened crenulated base and the rising squarish peristome in a very young colony. Considerable modification occurs in older zoids (see Figs. 15, 17, 18).

Figure 14. Detail of Circle Y of Fig. 3, shows a young flexible zoid beginning its upward growth. Drawn to Scale Y.

Plate IV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The genus Nolella (formerly Cylindroecium) contains a number of species, most of which differ from *Nolella blakei* in tentacle number (see Table II) *Nolella alta* and *Cylindroecium pusillum* both have about 10 tentacles. However, *Nolella alta* differs from *Nolella blakei* in having wider and longer zoids These are about double or more the width and in some instances ten times as long as those of *Nolella blakei*. *Cylindroecium pusillum* (Hincks, 1880a, pages 537 to 538) differs slightly in appearance of the expanding "stolon" as it approaches the zoid base, being somewhat more like a small Victorella in that respect than is *Nolella blakei*. Hincks gives very little data on it *Nolella blakei* differs from *Nolella sawayai* (Marcus, 1938, Plate XII, Fig. 30) in bodily proportions and cuticular outgrowths. Erect sawayai zoids are about as long but about twice as wide as those of blakei. Also, encrusted cuticular processes jut out in all directions from the upright tube and peristome in sawayai but not in blakei.

O'Donoghue incompletely described and figured three Nolella (Cylindroecium) species. *C. repens* (1923, page 50); *C. spinifera* (1924, Plate IV, Fig 27, page 59) and *C. horridum* (1926, p. 61), but gave no measurements or tentacle numbers. His repens had basal processes but the zoids tapered too sharply from base to tip, like a wedge or sugar beet. His spinifera and horridum had numerous spines about the lower part of the peristome, so were quite unlike *Nolella blakei.*

In summary, *Nolella blakei* resembles in one way or another a number of Nolella, Arachnoidea and Arachnidium species but differs from most in tentacular number and from some species in other characteristics as growth habit, body proportions, relative size, etc.

Summary

Perophora viridis, collected from Martha's Vineyard, Mass., and brought into the laboratory for culturing, yielded a bryozoan, *Nolella blakei* n. sp., which was cultured and observed alive for a time *Nolella blakei* is an intermediate form whose young zoids resemble those of Arachnoidea and Arachnidium but whose older zoids are definitely Nolella. It was erected a new species on the basis of its tentacle number (8 to 12) and general zoarial and zooecial characteristics. It increases to 88 the number of bryozoans known from the general Woods Hole region.

Plate IV

Figure 15. An older zoid, probably not full grown, shows the changed proportions of base to upright part of zoid. The digestive tract and zooecial tube are greatly elongated. The basal crenulations are absent. Labels: (B) rectum; (C) intestine; (E) esophagus; (F) stomach; (M) muscles; (N) squared peristomeal orifice; (P) gizzard? or proventriculus?; (S) stolon; (V) vestibule. Drawn to Scale Z.

Figure 16. An enlarged view of the two zoids of Figure 4 (P) as they looked after seven days' growth. The stolons between them are short. Drawn to Scale Y.

Figure 17. An older retracted zoid with a long gut. Labelled as in Figure 15, including tentacles (T). Drawn to Scale Y.

Figure 18. Another older zoid showing a stolon (S), the exceptionally long and narrow esophagus (E) and rectum, the partial extrusion of the collar (U) and the tentacles (T). Drawn to Scale Y.

Figure 19. A very young basal enlargement showing the anlage of the future polypide in the center. It was debris-covered and hence difficult to study. Labels: (D) distal part; (R) septum; (W) proximal extension of zoid. Drawn to Scale Y.

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X-RADIATION OF EGGS OF RANA PIPIENS AT VARIOUS MATURATION STAGES [1]

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INTRODUCTION

Interference with the normal course of embryological development has been the basis for many studies in experimental embryology. One of the most useful methods toward this end has been the study of abnormalities produced by various types of irradiation.

Many earlier investigators (Bohn, 1903; McGregor, 1908; Bardeen, 1909; O. Hertwig, 1911; G. Hertwig, 1911, 1912; P. Hertwig, 1916), working with various types of gametes and embryos, attempted to ascertain the effects produced by treatment with x-rays and radium. As stated by Butler (1936), however, "The work which has been done in this field has been, for the most part, qualitative in nature, and in many cases, particularly in the earlier investigations, the qualitative results are not thoroughly trustworthy. In many cases the amount of radiation which reached the egg or embryo was either unknown or at least unstated, the area of the embryo which came under the influence of the radiation was undetermined and little attention was given to the influence of external factors other than radiation, such as change of temperature or chemical changes in the medium." Since the standardization of the roentgen unit at the Fifth International Congress of Radiation in 1937, work in the field has been aimed at a more controlled study of the effects of accurately controlled doses of irradiation on adequate numbers of the various embryological stages.

The purpose of the present investigation was to make a study of the effects of x-radiation on the eggs of *Rana pipiens*. Since, in *Rana pipiens*, the ovarian eggs possess germinal vesicles and the uterine eggs are in the metaphase of the second maturation division, it was possible to study the effects of the x-radiation at two different maturation phases. A comparison was also made with eggs irradiated shortly after fertilization when the second polar body is being formed. It was thought, too, that a comparison of x-radiation studies on the eggs of *Rana pipiens* with the x-radiation studies of Rugh (1939) on the sperm of the same species would prove of value.

I wish to express my gratitude to Professor Roberts Rugh [2] for suggesting this problem and for his guidance through the course of the work, and to Dr. Titus C. Evans of the Radiological Research Laboratory, Columbia University, for so graciously making available the x-ray facilities.

[1] A dissertation in the Department of Biology submitted to the Faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

[2] Present address: Radiological Research Laboratory, College of Physicians and Surgeons, Columbia University, New York, N. Y.

Materials and Methods

Rana pipiens were obtained during their period of hibernation, from October to May, from Alburg, Vermont, and were kept on a water table in running water in the laboratory. Eggs were obtained by the injection of pituitary glands and fertilized according to the method of Rugh (1948).

Irradiation was carried out by means of two Coolidge-type water-cooled tubes at 180 kv. peak and 30 milliamps. Filters consisting of two mm. of copper plus one mm. of aluminum were used for all except the highest dose of 41,600 r. which was unfiltered. The intensity for the doses ranging from 100 r. through 1600 r was 50 r./min. at a target distance of 36 cm.; that for doses ranging from 3200 r through 25,600 r. was 675 r./min. at a target distance of 9.8 cm.; that for doses of 41,600 r. was 4150 r./min. at a target distance of 9.8 cm. The temperature ranged from 26° C. to 27° C.

It was necessary that the method of administering the irradiation vary slightly for the eggs at different stages of maturation.

(1) The ovarian eggs were treated by x-raying the female donor in the region of the ovaries. The females were then injected with pituitary glands and forty-eight hours later the eggs were stripped into a normal (unirradiated) sperm suspension. As soon as the jelly coating swelled, the eggs were separated into groups of from three to five. Following first division of the zygote, cleaved eggs were separated from uncleaved, and the eggs which had cleaved were placed in tanks measuring 24 in. × 12 in. × 6 in. in four inches of tap water at 67° to 70° F. in which constant aeration was maintained. Lettuce and Elodea were supplied as food material after the animals had hatched. Eggs from unirradiated females handled in the same way were used as controls.

(2) For the study of uterine eggs, female frogs were injected with pituitary glands forty-eight hours prior to irradiation to bring the eggs down into the uteri. After the removal of a few eggs into normal sperm suspension as controls, the females were irradiated in the region of the uterus. In this group it was possible to strip some eggs into sperm suspension after the administration of each irradiation dose so that one female could be used to provide eggs for all dosage levels. The same procedure was then followed as has been described for the ovarian eggs.

(3) A few studies of the effect of x-radiation on fertilized eggs were made with unfiltered radiation from a water-cooled Coolide-type tube at 185 kv. peak and 25 milliamps.[3] In these studies, normal eggs were stripped into dishes of normal sperm suspension and the dishes of eggs were irradiated with doses from 33 r. to 1000 r. approximately twenty minutes after insemination (at the time when the second polar body was being formed). After irradiation, the eggs were handled as described above.

Counts were made of cleaved eggs, gastrulae, neurulae, hatched tadpoles and normal tadpoles at each dosage level. Counts of gastrulae included all those individuals that showed a dorsal lip of the blastopore. Counts of neurulae included all those individuals that showed elongation and evidence of neural folds.

[3] The x-ray facilities for this part of the work were made available through the kindness of Dr. R. S. Anderson of Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York.

The percentages for each stage were calculated on the basis of the preceding stage in order to eliminate the possibility that the percentages for each of the various stages would reflect the effects of the irradiation on the previous stage.

Observations

Jelly of irradiated ovarian and uterine eggs

Damage to the jelly surrounding the eggs, such as has been reported for the jelly of the eggs of Arbacia (Evans, Beams and Smith, 1941) following irradiation was not observed except on the ovarian eggs subjected to the highest doses (25,600 r, and 41,600 r.). The jelly on the eggs from some of the animals receiving these high doses was opaque, flaccid and watery. Animals producing such eggs were found upon autopsy to have very shrunken oviducts and hemorrhagic uteri much distended with jelly. Because of this fact, and the fact that there was no noticeable effect on the jelly of the uterine eggs which were irradiated after they had traversed the oviduct, it is thought possible that the effect noted on the jelly of the ovarian eggs was an indirect one, caused by irradiation damage to the oviducts.

Cleavage of irradiated ovarian and uterine eggs

The cleavage rate and pattern were the same in the irradiated as in the control eggs, although a considerably higher percentage of ovarian eggs than uterine eggs cleaved at each x-ray dose as can be seen from Table 1 and Figure 1. In each of

TABLE 1

Cleavage

X-ray dose in roentgen units	Ovarian eggs			Uterine eggs		
	No. eggs	No. cleaved	%	No. eggs	No cleaved	%
Control	780	750	96.15	526	469	89.16
100 r.	400	392	98.00	346	292	84.39
200 r.	936	909	97.11	500	386	77.20
400 r.	409	400	97.80	696	492	70.70
800 r.	498	352	70 70	742	385	51.88
1,600 r.	1,190	815	68.48	796	394	49.49
3,200 r.	1,043	912	87.44	742	393	52.96
6,400 r.	1,036	811	78.28	696	454	65.22
12,800 r.	523	400	76.50	785	361	45.98
25,600 r.	2,321	1,408	60.66	833	96	11.52
41,600 r.	1,661	56	3.37	500	0	0

the two groups, the percentage of cleavage drops off with increasing irradiation (though more gradually in the ovarian curve), rises slightly, and again drops until at 41,600 r. only 3.4 per cent of the ovarian eggs cleave and none of the uterine eggs cleave. Thus these curves show a slight "paradoxical effect" (Dalcq, 1930) or an inverse relationship between the radiation dose and the damage produced.

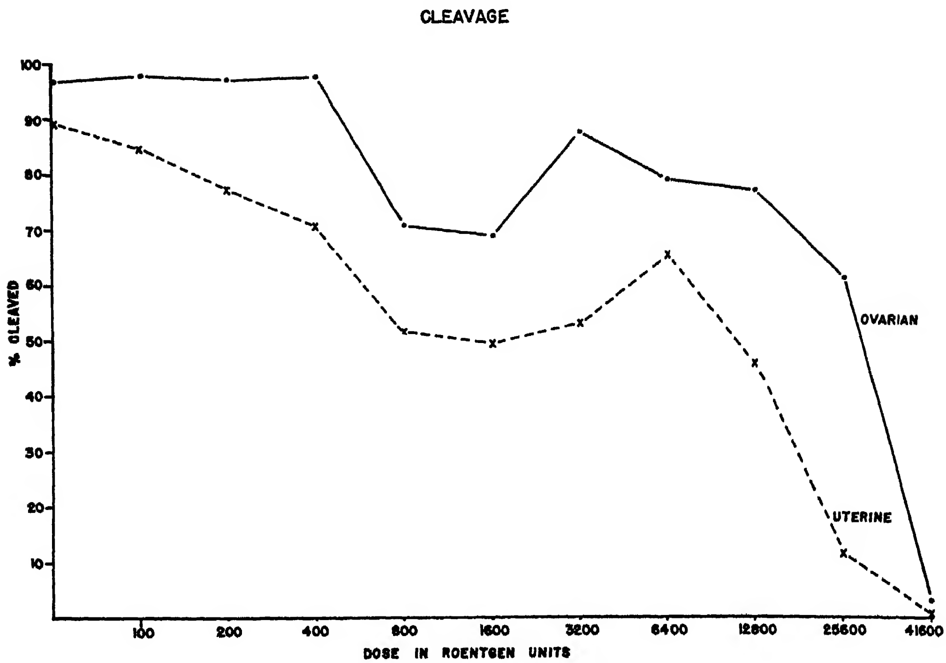


FIGURE 1.

Gastrulation of irradiated ovarian and uterine eggs

It appears from Tables 2 and 3 and Figures 2 and 3 that irradiation with x-rays even at high doses does not affect appreciably the ability of either the ovarian or uterine eggs to undergo morphogenetic movements at least up to the formation of a dorsal lip. The number of abnormal gastrulae, however, markedly increases with

TABLE 2

Ovarian eggs

(Each percentage based on previous stage)

X-ray dose in roentgen units	No. eggs cleaved	Per cent Gastrulated	Per cent Neurulated	Per cent Hatched	Per cent Normal
Control	780	99.20	99.19	100	99.59
100 r.	400	99.25	100	99.75	97.73
200 r.	1,600	98.25	98.22	90.28	87.73
400 r.	800	99.75	100*	99.00	92.41
800 r.	3,669	97.66	96.60	96.62	48.53
1,600 r.	782	100	98.72	93.99	26.09
3,200 r.	1,600	97.00	90.59	70.27	28.34
6,400 r.	1,182	97.12	81.45	60.43	30.27
12,800 r.	520	96.92	95.63	64.94	16.61
25,600 r.	935	94.33	78.23	46.23	19.12
41,600 r.	56	87.50	71.43	17.14	0

TABLE 3

Uterine eggs

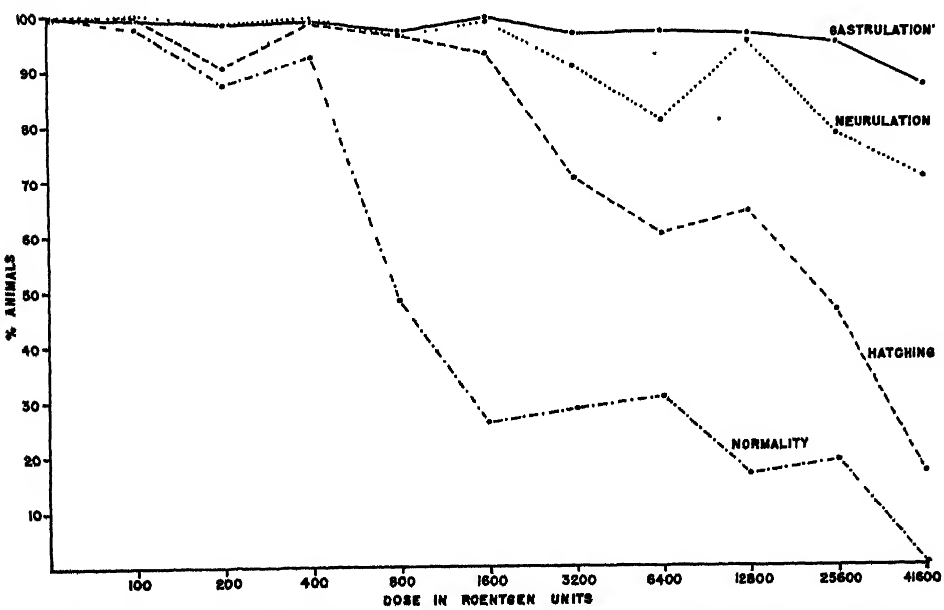
(Each percentage based on previous stage)

X-ray dose in roentgen units	No. eggs cleaved	Per cent Gastrulated	Per cent Neurulated	Per cent Hatched	Per cent Normal
Control	825	98.66	97.30	97.10	97.79
100 r.	554	97.47	97.59	97.15	80.47
200 r.	606	98.18	98.82	84.69	49.80
400 r.	860	98.48	94.21	76.44	10.98
800 r.	676	97.92	93.35	50.97	0.32
1,600 r.	612	91.66	90.73	49.12	5.60
3,200 r.	791	98.86	91.18	63.81	5.49
6,400 r.	758	97.09	90.48	64.26	4.21
12,800 r.	600	94.83	90.69	64.53	2.70
25,600 r.	198	94.94	77.66	46.58	4.62
41,600 r.	0	0	0	0	0

increased irradiation. Since at a dosage of 41,600 r. none of the uterine eggs cleaved, no further data could be obtained on these eggs at this dosage.

The types of abnormal gastrulae produced are very similar in all groups. The severity of the abnormalities, however, increases with increasing dosage and is more

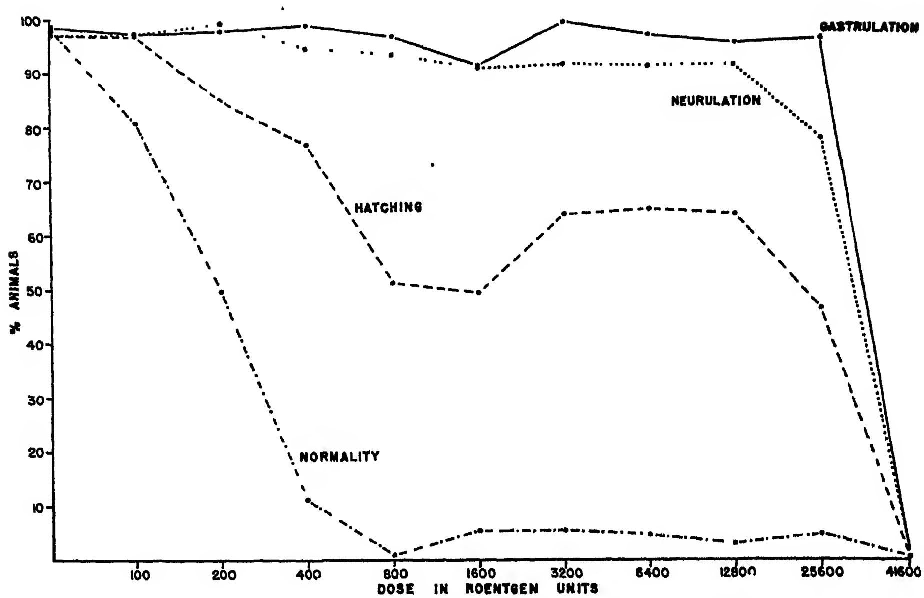
EFFECT OF X-RAYS ON OVARIAN EGGS



For each point on each curve, the percentage is based on the number of animals which reached the preceding stage of development

FIGURE 2.

EFFECT OF X-RAYS ON UTERINE EGGS



For each point on each curve, the percentage is based on the number of animals which reached the preceding stage of development

FIGURE 3.

marked in most of the uterine batches than in the corresponding ovarian groups. There are those blastulae which gastrulate apparently normally through the formation of a dorsal lip and then cytolyze. Some form both dorsal and lateral lips, and some develop abnormally large yolk plugs of various sizes. In all of these groups, it seems evident that epiboly continues without the inturning of material, since many of the gastrulae have irregular, "warty" surfaces. Some exogastrulae are also found. Many of the animals which continue growing, but do not neurulate, take on extremely irregular and amorphous forms.

Neurulation of irradiated ovarian and uterine eggs

From Tables 2 and 3 and Figures 2 and 3 may be seen the percentages of those embryos which neurulated, based on the number that gastrulated. It is evident that a very large percentage of those animals which gastrulate also neurulate. Of the embryos that neurulated a fairly high percentage was normal in the ovarian group except at the two highest doses. In the uterine group, however, there were fewer normal neurulae.

There are numerous types of abnormal neurulae found in the irradiated groups of animals. These range from those which go no further than slight elongation and flattening of the dorsal surface to those with slightly incomplete posterior closure of the folds because of the persistence of a yolk plug. Included in the group are those which form just the suggestion of short, low neural folds; those which form only one fold; those which form well-defined folds which do not close; and those

which form folds which close only partially. Most of these abnormal forms have persistent yolk plugs.

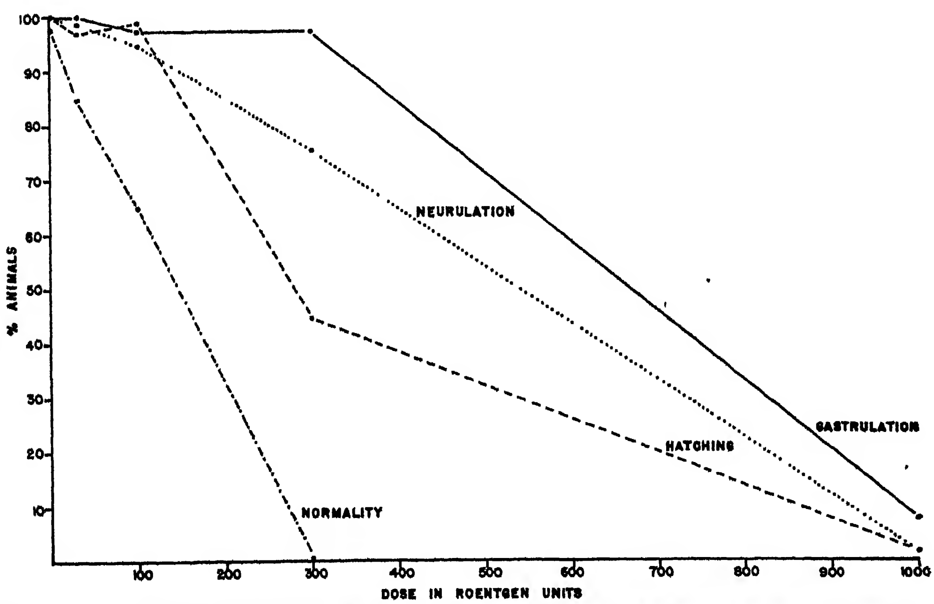
Hatching of irradiated ovarian and uterine eggs

The number of animals hatching from irradiated ovarian eggs (Tables 2 and 3 and Figures 2 and 3) declines with increasing dosage while the hatching curve for uterine eggs shows some evidence of a "paradoxical effect". It should also be noted that, here again, the lower irradiation doses cause a more rapid initial drop in the uterine than in the ovarian curve.

Among the hatched tadpoles, there are various abnormal forms. Again, it is evident that the animals developing from eggs which were x-rayed in the metaphase of the second maturation division (eggs in the uterus) are more abnormal (Plates 3 and 4) than those developing from eggs x-rayed in the germinal vesicle stage (eggs in the ovary) (Plates 1 and 2). Surprisingly enough, a few of the animals developing from ovarian eggs which had been given a dose of 25,600 r. appear quite normal (Plate 2). Among the tadpoles from each group, however, are found some microcephalics; some with spina bifida; some stubby, curved, typically haploid appearing; some with short, crumpled, curled, notched or clipped tails; some with papillated surfaces; some edematous and some of quite amorphous shapes with large remaining yolk plugs or yolk masses exposed.

In Tables 2 and 3 and Figures 2 and 3 are seen the percentages of those animals which hatched which were normal in appearance. The ovarian curve again is

EFFECT OF X-RAYS ON FERTILIZED EGGS



For each point on each curve, the percentage is based on the number of animals which reached the preceding stage of development

FIGURE 4.

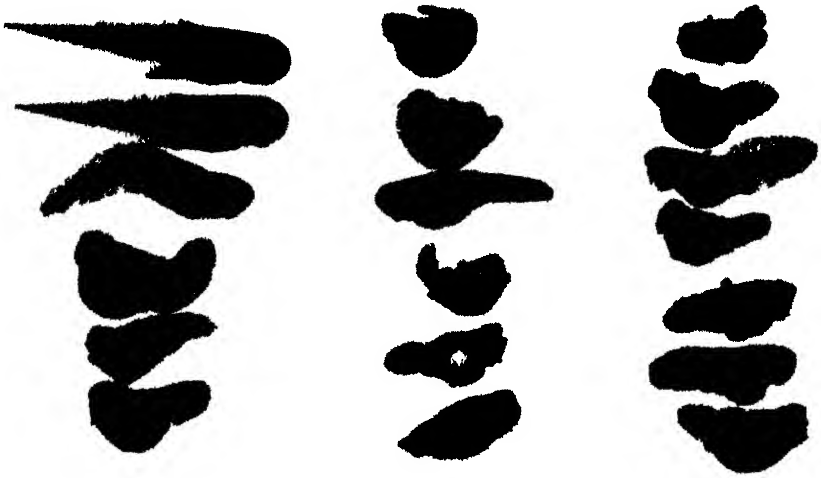
PLATE I

CONTROLS

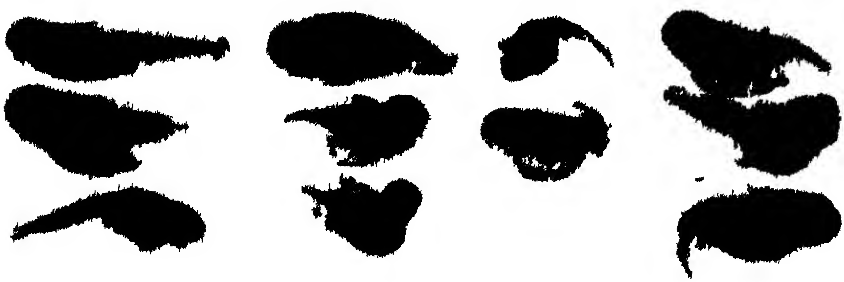
OVARIAN EGGS EXPOSED TO 400 ROENTGEN UNITS

OVARIAN EGGS EXPOSED TO 1600 ROENTGEN UNITS

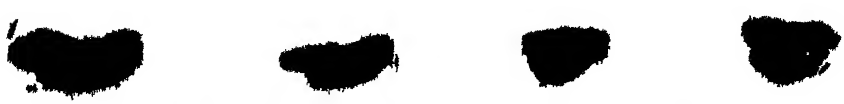
PLATE 2



OVARIAN EGGS EXPOSED TO 6400 ROENTGEN UNITS

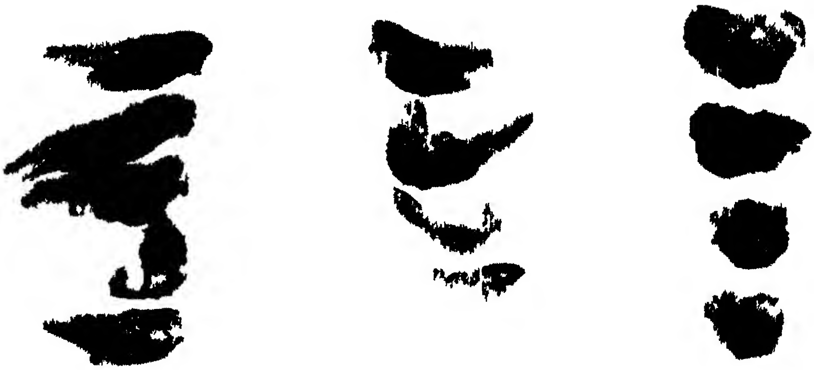


OVARIAN EGGS EXPOSED TO 25600 ROENTGEN UNITS



OVARIAN EGGS EXPOSED TO 41600 ROENTGEN UNITS

PLATE 3



UTERINE EGGS EXPOSED TO 400 ROENTGEN UNITS

UTERINE EGGS EXPOSED TO 1600 ROENTGEN UNITS

PLATE 4

UTERINE EGGS EXPOSED TO 6400 ROENTGEN UNITS

UTERINE EGGS EXPOSED TO 25600 ROENTGEN UNITS

higher than the uterine curve. There seems to be a gradual effect on the ovarian eggs at dosages of 100 r. through 400 r. This is followed by a rapid drop to 1600 r. and then a very gradual decline. The effect on the uterine eggs is, however, somewhat different. Here there is a very rapid drop to 800 r. followed by an extended very low plateau.

Thus it may be seen that the effect of the irradiation has, in general, been less on the ovarian eggs.

Irradiation of fertilized eggs

The results of irradiating fertilized eggs which were in the process of giving off their second polar bodies may be seen in Table 4 and Figure 4. It is evident that a dose of 100 r. allows most of the eggs to gastrulate, neurulate, and hatch. At this dosage approximately 65 per cent of the resulting tadpoles are normal in appearance. However, at 300 r. there is a marked drop in the number which neurulate and hatch, and none of the animals is normal. At a dosage of 1000 r. some of the eggs gastrulate, but none goes on in development. The types of abnormalities seen are much the same as those resulting from irradiation of the eggs alone.

TABLE 4

Fertilized eggs

(Each percentage based on previous stage)

X-ray dose in roentgen units	No. eggs cleaved	Per cent Gastrulated	Per cent Neurulated	Per cent Hatched	Per cent Normal
Control	200	100	100	100	97.50
33 r.	208	100	98.08	97.06	84.34
100 r.	190	97.37	94.59	98.86	64.74
300 r.	452	97.57	75.74	44.31	0
1,000 r.	250	9.60	1.43	0	0

Delayed fertilization

Since fertilization can be accomplished only after the eggs have traversed the oviduct, it was necessary, in the case of the ovarian eggs, to delay a minimum of twenty-four hours between irradiation and fertilization (i.e., the length of time necessary for stimulation by the pituitary glands to bring the eggs from the ovary to the uterus). Since recovery from irradiation has been reported (Packard, 1930; Henshaw, 1932; Evans, 1934, et al.), it was decided to test whether the lesser effect of the irradiation on the ovarian eggs was the result of recovery of the eggs during the period of delay rather than a lower susceptibility of the ovarian eggs. Therefore a few batches of uterine eggs were delayed for a period of forty-four hours between irradiation and fertilization. The results of such a test are indicated in Table 5. (Eggs from the same female were used for both batches.)

It is evident from this that the uterine eggs, at least, do not seem to recover from irradiation effects if a period of time is allowed to elapse between irradiation and fertilization. The general trend seems to be in the other direction, i.e., the delay results in slightly poorer development.

TABLE 5

Delayed fertilization

	Fertilization immediately after irradiation	Fertilization delayed 44 hours after irradiation
Per cent Gastrulated	99.18	96.42
Per cent Neurulated	95.90	83.92
Per cent Hatched	63.52	48.21
Per cent Normal	1.22	5.35

High intensity effects

For the sake of convenience, the irradiation intensity differed at the low and high doses, as was mentioned above. Thus the irradiation administered for doses 100 r. through 1600 r. was at the rate of 50 r./min. while that administered for doses 3200 r. through 25,600 r. was at 675 or 685 r./min.

To test whether this intensity difference was affecting the results, as has been reported by some other workers (Forssberg, 1933; Sax, 1939), some uterine eggs were given a total dose of 800 r. at the rate of 675 r./min. The results of that test compared with the average at the lower intensity at a total dose of 800 r. are shown in Table 6.

TABLE 6

The effect of different intensities of x-radiation

	800 r. at 50 r. per min.	800 r. at 675 r. per min.
Per cent Gastrulated	97.92	99.18
Per cent Neurulated	91.42	95.90
Per cent Hatched	46.60	63.52
Per cent Normal	1.48	1.22

It is evident from this test that irradiation at a higher intensity is not more damaging than that at a lower one. Thus the intensity factor does not seem to be involved in this work. The formula D = TI (Dose = time × intensity) seems to be valid for this work.

DISCUSSION

The significance of the foregoing results will be better understood if they are compared in the light of previous research.

A study of the sensitivity to x-radiation of the stages of maturation of the eggs of *Rana pipiens* has been made in the present work. Many attempts have been made in the past to test the difference in susceptibility to irradiation between resting cells and those in varying phases of meiosis or mitosis. Most of the results, from widely diverse groups of plants and animals, have pointed at least to the fact that a cell undergoing division is more sensitive than is a resting cell (Giese, 1947). It was possible in the present work to test resting cells (ovarian eggs), cells in metaphase of the second maturation division (uterine eggs) and those completing the second maturation division (fertilized eggs). The results show quite clearly that the cells undergoing maturation division are the more susceptible to damage by x-rays. The fertilized eggs were obviously the most sensitive, but the studies on these eggs were complicated by the necessity of irradiation of the sperm along with the eggs.

The differences in susceptibility between resting and dividing cells have been explained by Heilbrunn and Mazia (1936) on the basis of the greater rapidity with which calcium released from the cytoplasm by the irradiation can reach the chromosomes when no nuclear membrane is present. Sparrow (1944) has correlated sensitivity with nucleic acid metabolism; the most sensitive stages being those which have a high content of desoxyribose nucleic acid or nucleotides.

It should be noted here that throughout the course of this work considerable variation in susceptibility of the eggs of the frog to the effects of x-radiation has been observed. Similar findings have also been reported following irradiation of the eggs of Triton (P. Hertwig, 1916) and Chaetopterus (Packard, 1918) and following treatment of the eggs of the frog with agents such as 2, 4-dinitrophenol (Dawson, 1938) and colchicine (Keppel and Dawson, 1939). One factor that may possibly be involved is the varying genetic constitution that would be expected in any wild population as judged from the work done on Drosophila (Dobzhansky, 1939). It is also interesting to note that inherited differences in sensitivity to ultra-violet rays and x-radiations have been reported in a strain of bacteria (Witkin, 1946, 1947). This variation coupled with work on small numbers of animals and lack of a standardized x-ray unit may be the reason for so many conflicting reports in the field.

It was thought that a comparison of the effects of x-radiation of the eggs of *Rana pipiens* with studies on the sperm of the same species (Rugh, 1939) would prove of value. Such a comparison shows that the sperm are more sensitive than are the eggs. However, the so-called "paradoxical effect," an inverse relationship between the radiation dose and the damage produced, reported after exposure of various gametes to x-rays, ultra-violet and radium radiations (O. Hertwig, 1911; G. Hertwig, 1911; Simon, 1930; Dalcq, 1930; Rugh and Exner, 1940), is not nearly so marked in the present work on the eggs as it has been shown by Rugh (1939) to be on the sperm. The cause of the paradoxical effect has been claimed, from cytological studies (G. Hertwig, 1911), to be a gradual destruction of the irradiated chromosome complement with increased dosage until, at the higher doses, development is haploid and is carried on under the sole influence of the normal chromosome complement. Dalcq (1929), however, is of the opinion that the paradoxical effect is only explicable in all its details by the intervention of the nucleoplasm. Probably a true paradoxical effect on the eggs is prevented by irradiation damage to the cytoplasm, and in the case of the ovarian eggs to a prevention of maturation of those eggs which are very badly damaged. That cytoplasmic damage does occur and does affect the nucleus has been shown by the work of Duryee (1939 a and b, 1947). It is obvious that no paradoxical effect would be possible in the eggs irradiated when the second polar body was being formed and that the x-radiation effects on these eggs would be much more severe, since in these studies both the egg and sperm have been irradiated.

The last part of the problem to be discussed is the effect of the irradiation on the mechanics of development. The lack of effect on cleavage rate and pattern found in the present work agrees with the results of Ancel and Vintemberger (1925) on Rana eggs in the two-cell stage, Dalcq (1929) on unfertilized Rana eggs and Rugh (1939) on *Rana pipiens* sperm. However, accelerated cleavage has been reported for fertilized amphibian eggs by other workers (Gilman and Baetjer, 1904;

Hoffman, 1922). Lack of controlled temperature may have been a factor in the latter work. There is some indication, too, that fertilized eggs subjected to very much higher doses of x-ray than were used in the present work will delay cleavage and alter the cleavage pattern (Langendorff and Langendorff, 1933).

Cleavage appears to be a critical stage for the x-ray damaged eggs to accomplish. It would seem that if the eggs cleave, at least some morphogenetic movements will take place in almost all of them. As has been mentioned, however, there is a fairly marked drop in the numbers of normal gastrulae.

A large percentage of those embryos which show any gastrular movements will also at least attempt neurulation, the amount of neurulation being dependent on how far gastrulation has progressed. The number of normal neurulae corresponds quite closely with the number of normal gastrulae. This seems to be a good indication that the normality of the neurulae is directly dependent on the normality of the gastrulae.

Not all the animals that appear to have undergone normal neurulation movement are able to hatch. This may be an indication of lack of a hatching enzyme or other fundamental cellular disarrangements. The number of normal tadpoles produced is obviously very much lower than the numbers of normal gastrulae or neurulae. It would thus almost appear that there are two major effects of the x-radiation; one immediate effect which inhibits cleavage and the other which becomes most apparent at the time of hatching. If the eggs cleave, a fairly high percentage continues development to become normal gastrulae and neurulae, and an even higher percentage shows at least abnormal gastrulation and neurulation movements. The second effect appears after neurulation in the producation of a high percentage of abnormal tadpoles. Some of these are the further development of abnormal neurulae, but many of them appear to develop from normal-appearing neurulae.

The abnormalities resulting from x-radiation of the eggs of *Rana pipiens* seem similar to those obtained by Rugh (1939) in x-radiation of the sperm of the same form. Similar abnormalities have also been obtained in amphibians by subjecting developing embryos to supra-maximal temperatures (Hoadley, 1938), to 2, 4-dinitrophenol (Dawson, 1938), by fertilizing over-ripe eggs (Witschi, 1930 and Zimmerman and Rugh, 1940), by placing fertilized eggs in colchicine solutions (Keppel and Dawson, 1939) and by subjecting fertilized eggs to hydrostatic pressure (Rugh and Marsland, 1943). Any of these agents may in some way (1) affect the surface tension of the cells and/or the pH of the blastocoelic fluid to prevent normal blastoporal invagination and the infolding of the neural plate (Holtfreter, 1943), (2) either completely or partially damage the ectoderm to prevent its normal spreading (Holtfreter, 1943) and/or (3) damage the endoderm to prevent its spreading and impair the polarity of the mesodermal cells (Holtfreter, 1944).

Although most of the agents, other than x-rays, probably damage the nucleus indirectly by first damaging the cytoplasm, while x-rays probably damage both directly (Failla, 1937), the abnormalities ultimately produced are similar.

Summary and Conclusions

1. Eggs of *Rana pipiens* in germinal vesicle stage (ovarian eggs), metaphase of the second maturation division (uterine eggs) and during completion of the second

maturation division (eggs twenty minutes after fertilization) were irradiated with doses of roentgen units ranging from 100 r. to 41,600 r. Counts were made of cleaved eggs, gastrulae, neurulae, hatched embryos and normal tadpoles (i.e., embryos beyond stage 25).

2. The jelly surrounding the eggs was apparently unaffected except on the ovarian eggs at the highest irradiation doses (25,600 r. and 41,600 r.). Damage at these doses may have been an indirect effect of the x rays on the oviduct.

3. The cleavage rate and pattern were not affected. The percentage of cleavage was very much lowered, however, by the irradiation.

4. The lower doses of x-rays had relatively little effect on the ovarian eggs. At a dose of 400 r., for instance, 97.8 per cent cleaved, 99.75 per cent gastrulated, 99.75 per cent neurulated, 98.75 per cent hatched and 91.25 per cent appeared normal.

5. The uterine eggs were more sensitive to x-radiation than were the ovarian eggs, while the fertilized eggs were by far the most sensitive of the three groups tested. Thus, when a dose of 1000 r. was administered to the fertilized eggs, only 9.6 per cent of the animals gastrulated, 1.4 per cent of these showed evidence of neurulation and none hatched. A dose of 41,600 r. inactivated the uterine eggs, while at this latter dosage, although only a small percentage of the ovarian eggs cleaved, of those that cleaved, 87.5 per cent gastrulated, 62.5 per cent neurulated, and 10.71 per cent hatched.

6. The types of abnormalities produced by irradiation of the eggs are similar to those resulting from irradiation of the sperm of the same species.

7. A delay of twenty-four hours between irradiation and fertilization of the uterine eggs, to simulate the required delay for the ovarian eggs, appeared to have no effect. Thus there seems to be no recovery from the effects of the x-radiation.

8. There is no difference in the effectiveness of the different x-ray intensities used in this work as long as the total roentgen exposure is the same.

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STATISTICAL AND PHYSIOLOGICAL STUDIES ON THE INTERPHASIC GROWTH OF THE NUCLEUS

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1. INTRODUCTION

1. (*a*) *The "rhythmic" growth of nuclear size*

We define as "rhythmic growth" that growth of the cell nucleus in which periods of quick development alternate with others of rest or slower growth. The volumes attained in each step of this growth are generally in a simple mathematical ratio.

The simplest rhythmic growth is that in which nuclear size is doubled at every step (Jacobj, 1926). By measuring the nuclear sizes (volumes) of a homogeneous tissue, such as, for instance, the mammalian liver, and distributing the values into classes of a frequency curve ("Statistiche-kariometrische Untersuchungen), Jacobj verified that there are many modal classes, and that these modes form a regular series with a ratio of 1 : 2.

Some studies following the discovery of Jacobj led to the explanation that rhythmic growth of the nucleus is due to the reduplication of the nuclear content, which establishes in the tissues a series of polyploid nuclei or nuclei with polytene chromosomes (D'Ancona, 1939–40–41; Biesele, Poyner and Painter, 1942). Biesele later published a series of papers dealing with the size of the chromosomes in different conditions, ages, and tissues. We cannot discuss these results here because they do not appear to be sufficiently defined in relation to nuclear volume and nuclear growth.

In the meantime, other studies revealed that the steps in rhythmic growth of the nucleus may occur at a ratio different from that of 1 : 2. To some authors this fact seems difficult to interpret in genetic terms (Wermel and co-workers). Several authors described a rhythmic growth with a ratio of 1 : 1, 5 (Wermel and Portu-

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galow, 1935; Bogojawlensky, 1935), and others found the ratio to be 1:1.41, which is the value of the square root of 2 (Brummelkamp, 1939 and G. Hertwig, 1938–39). The nature of these intermediate stages, which German-speaking authors call "Zwischenklassen," has been discussed from different points of view in a previous paper (Schreiber, 1943). Both Brummelkamp (1939) and Hertwig (1938–39) studied the problem from a physiological point of view without considering the genetic basis of the phenomena that form nuclear activity during the interphase.

The main object of the present paper is the study of the intermediate classes of nuclear size during the reduplication step.

For this purpose, statistical analysis of nuclear size has been used, as by previous authors, in actively reproducing tissues as well as contemporary physiological methods such as hormone stimulation. With this method we can arrest or increase the mitotic activity of the cells by specific stimulation, and thus define the limits of the interphasic variation of nuclear size.

For the statistical study of nuclear growth some basic facts must be ascertained. First we must find out whether there is any correlation between nuclear volume and multiple value of the genome,[2] and if so at what point in the nuclear cycle it occurs.

The problem as to whether the number of chromosomes is related to the volume or to the surface of the nucleus, or to some other quantitative value of the cell is still open to discussion.

The second fact to be established is the limit of the variation of nuclear size during an interphasic growth cycle, with reference to the volume of a nucleus with a genome of known quantitative value. The term "interphase" is here used to indicate in a general way the period between two somatic divisions. Although the term was originally used to signify the interval between the first and second meiotic divisions, it was subsequently used by various authors as a synonym for "resting" or "metabolic" stage.

Another important fact which must be established in these caryometric studies is whether modifications of the model value of nuclear size in a tissue are natural or experimentally induced. We must be sure that variations in mode represent true growth or reduction and are not due to the occurrence of various types of cells of different characteristic sizes (i.e. grade of ploidy) originally present in the tissue and assuming by differential proliferation, the role of the main cell under the new conditions (Schreiber and Romano-Schreiber, 1941; Paccagnella, 1944–45).

In previous papers we approached these different problems from different angles and with different materials, each offering characteristics that would answer one of the questions. Thus the problem of the relationship between nuclear size and

[2] The term "genome" is here used following the definition of Sharp (Introduction to cytology, 3d Ed., New York, pg. 121): "In any given kind of plant or animal each nucleus contain an outfit or complement of chromosomes composed of a certain number of members showing characteristic difference in form and function. As a general rule the nucleus of an egg before fertilisation contains a complement made up of one each of several kind of chromosomes, such a complement is called a *set* or *genome*"; and pg. 353: "The monoploid chromosome set *or genome* is a group of chromosomes differing among themselves in the number and kind of their component elements. Ordinarly all or nearly all of the genetic elements (genes) are probably necessary to the normal activity of the nucleus; in other terms the genom is a harmonious differentiated system of elements, the majority of which are essential parts of the system."

genome has been studied in a series of polyploid Coffea plants (Schreiber, 1946) and in the spermatogenesis of snakes (Schreiber, 1946–47). The problem of the nature of the steps in rhythmic growth, and the limits of the mitotic interphase has been studied in experiments on the uterine cells under different physiological conditions and on the granulosa layer and luteal cells of the mammalian ovary (Salvatore and Schreiber, 1947; Schreiber, Mello and Salvatore, 1948; Salvatore, 1948).

We shall here summarize those studies which contribute to the knowledge of nuclear growth during interphase.

2. The Study of the Nuclear Growth

2. (*a*). *Statistical methods*

Analysis of the statistical distribution of nuclear sizes in a homogeneous mass of cells of some tissues verifies the existence of various modal values. This indicates that the nuclei stop growing or grow at a slower rate when they attain the sizes corresponding to the modal values. Within a given time, therefore, these nuclei appear with greater frequency. This finding is confirmed by comparing rhythmic growth of the nucleus, as indicated by caryometric statistical research, with the rhythmic growth of the nucleus in cells in cultures, measured at regular intervals with motion pictures (Wermel and Portugalow, 1935). A somewhat similar comparison between a statistical study of a dynamic phenomenon and the direct study of the same in living cells has been made by Mollendorff and co-workers (1937) in order to determine the relative length of the mitotic phases (see also W. Thompson-D'Arcy, 1942).

Some technical precautions must be taken, the first being that of geometric determination of nuclear size. In all tissues with spherical nuclei the problem is of course easy, but when we deal with ellipsoid-shaped nuclei the problem of the orientation of the axes is of the greatest importance and needs careful previous control. For the first type of cells the liver, testicle, and corpus luteum offer very good material; for the second groups the root tip cells, the cubical or cylindrical epithelia, and the smooth muscular cells of the uterine wall are very suitable.

All studies summarized here were conducted by drawing the nuclear outline with a camera lucida and measuring its diameters. The frequency curves were drawn and the modal values calculated. Since the curves are influenced by the growth of the nuclei they are not of the true "normal" type; mean, standard deviation, and median have no biological significance. Only the modal value is of biological interest because it reveals the steps during the growth cycle. The modal value of the volume can be calculated directly from the modal value of the diameter, which is not the case for other statistical parameters.

2. (*b*) *Physiological methods*

In the statistical study of nuclear variability, frequency curves sometimes have more than one modal value. Sometimes these modes are represented by very different frequencies, one mode appearing as principal and the others as secondary. If observed on isolated histograms the secondary modes may sometimes be considered statistically doubtful because they are determined from very scant data. However, if we consider the histograms of the same tissue under different physiological

conditions, we can frequently observe that what is a secondary mode under one condition may became a fundamental one in another physiological status.

Considering the histograms of various physiological conditions as a whole, we can not only confirm the statistical consistency of the small secondary modes of each physiological status, but also interpret the cyto-physiological significance of the nuclei that constitute each mode. This system of studying the caryometric variability of a tissue physiologically enables us to give biological value to certain data, which the purely statistical study of a single tissue could not do.

The modes that correspond to the volumes of the prophase are particularly important. These volumes belong to nuclei whose genes—and therefore chromosomes or chromonemata also—have completed a duplicating cycle and effectively represent a basic stage of interphasic growth, even though the prophasic nucleus shows some conditions of variability that affect caryometric measurements more than the interphasic nucleus (ellipsoidic form not oriented, larger imbibition phenomena, etc.).

The fundamental observation of Jacobj that the values of the maximum of frequency of nuclear sizes are in the relation 1:2:4:8, etc., leaves no doubt that the material constituents of the nucleus reduplicate at each cycle of growth, and therefore the phenomenon is related to the process of reduplication of the genes which constitutes the basic occurrence of the interphasic period. The nuclei belonging to the higher multiples of the volume are polyploid or with polytene chromosomes (Biesele, Poyner and Painter, 1942).

In our studies we are attempting to extend knowledge of the phenomena in the following manner:

First we tried to find out whether the nuclei that have no interphasic growth and have differing numbers of chromosomes (such as the meiotic elements) have a corresponding volume for each number of chromosomes.

Secondly we tried to ascertain whether the interval of rhythmic growth during which the nucleus duplicates its volume corresponds to an interphasic growth period, which begins with the post-telophase of the preceding division and ends with the prophase of the subsequent one.

Thirdly we tried to use special physiological conditions which would arrest nuclear reproductive activity and then cause it to begin again simultaneously in all nuclei under new, experimentally controlled conditions.

The statistical variability of these nuclei suggests that the growth obeying these physiologically stimulating or arresting conditions is of the "rhythmic" type.

With these three elements—comparison between nuclei without interphasic growth and with different numbers of chromosomes, comparison between the stages of rhythmic growth and the prophases, and the induction of simultaneous interphasic growth in all the nuclei of a tissue by means of controlled physiological stimulation—we can give a true significance of rhythmic growth in terms related to the duplicating processes of the nuclear genes.

2. (*c*) *The caryometric analysis of spermatogenesis*

The first question to be settled is whether in cells which have no interphasic growth and have a known but different numbers of chromosomes, the nuclear volumes have a constant value in proportion to the number of chromosomes.

The spermatogenetic series gives favorable results here, since the spermatocyte of the first order, the spermatocyte of the scond order, and the spermatid generally have chromosomes (or genomes) that are in the ratio 4:2:1.

The corresponding volumes of their nuclei, as has been known since the first studies of Jacobj (Jacobj, 1926; Freerksen, 1933; Hertwig, 1933; Sauser, 1936; etc.), are strictly in the same relation 4:2:1.

In certain cases the relationship is not the same (Wermel: Lepidoptera; Hertwig, Schreiber: Vertebrata), and we must consider these cases separately because they probably have different stages of endomitotic growth or some phenomena of chromatin elimination.

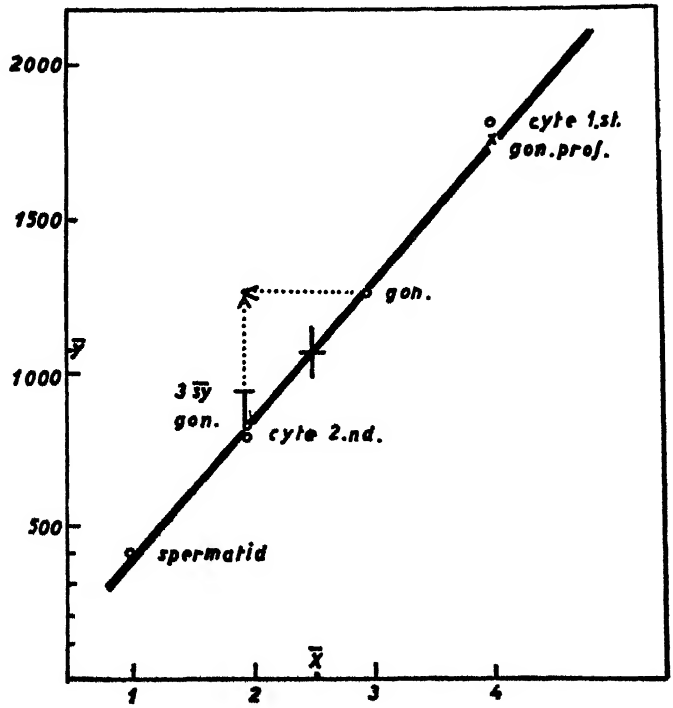

FIGURE 1. Scatter diagram and regression line between modal values of nuclear volume of spermatogenetic stages and the theoretical multiple values of the genome. From the average of 15 species of snakes.

It is important to stress here that the meiocytes ready for division represent a series of nuclei in which the multiple value of the genome is entirely proportional to the multiple value of the nuclear volume.

In the course of studies on meiocytes of snakes (Schreiber, 1946–48) we have verified this fact in 15 different species of neotropical Ophidia. The correlation between nuclear value and multiple value of the genome is always perfect, and the differences between the theoretical values and the actual ones are statistically insignificant.

We can therefore accept in testicular tissue the meiocyte series as a standard series of nuclear sizes that allows us to determine the quantitative value of the genome on the basis of nuclear volumes. This fact must be recognized as being limited to this tissue and no generalization made, for there are conditions in other tissues and organisms under which the correlation between genome and nuclear size does not follow the same rule (Wettstein, Dobzhansky, Barigozzi: see Schreiber 1946 c).

In the testicle, however, we find another category of cells, the spermatogonia, closely related to the meiocytes, but presenting a mitotic reproductive cycle with normal interphasic growth before the beginning of the meiotic process.

It is not our present task to analyze the growth phase which transforms the spermatogonium into a spermatocyte of the first order; these facts have been studied caryometrically in a short paper (Schreiber, 1948).

We will here analyze the mitotic cycle of the spermatogonium from the caryometric point of view by comparing the sizes attained by the nuclei of the spermatogonium during the interphase with the volumes just analyzed, of the meiocytes considered as standard size.

TABLE I

Nuclear volume in spermatogenesis (modal values)—average from 15 species of snakes

Cell	Spermatid	Spermatocyte 2nd order	Spermatogonium			Spermatocyte 1st order
			1st mode	2nd mode	Prophas	
Genome	n	2n	2n	(3n)	4n	4n
Volume	414	884	824	1271	1824	1765

The histogram of the nuclear volumes of a spermatogonium is generally unimodal, but in some cases presents a secondary modal value. These two modes are in a constant relative position, and from the study of many histograms (of 15 different species) we can conclude that the lower modal value corresponds to the volume of the second order spermatocyte, and hence to a resting diploid nucleus. The higher mode, which is generally the main mode, having a higher frequency value than the other corresponds to a volume that is 1.5 times the volume of the first mode, i.e. half way between the mode of the spermatocyte of the second order and the spermatocyte of the first order.

In the regression line between the modal values and the number of genomes (Fig. 1), the main mode of the spermatogonium corresponds to a genome value of 3. The difference between the volume of the main mode of the spermatogonium and the mode of the resting diploid nucleus (secondary spermatocyte) is statistically significant (more than 3 Sy.).

The volume of the spermatogonial prophase, which represents the final stage of interphasic growth in the mitotic cycle, is very nearly the same as that of the first order of the spermatocyte.

With these indications we can try to consider the entire interphasic growth of the spermatogonium (from the caryometric point of view) as follows: the diploid

nucleus grows during the interphase and reduplicates its volume when it reaches the prophase. During growth, however, it stops when it reaches a volume that is about one and a half times the initial one.

This relation (1:1.5) between the two modal values of the spermatogonial growth cycle corresponds to the "Zwischenklassen" which some authors (Brummelkamp,, Hertwig) describe in comparing the nuclear volumes of different cells, tissues, or species. In the case of the spermatogonium we can be sure that these intermediate classes in the statistical analysis belong to the growth phases of a single category of cells, as shown in the growth curves studied by Wermel and Portugalow in the motion pictures of cells cultivated in vitro. In our preceding papers we called this intermediate stage of interphasic growth—"sesquiphase."

The rise of common hystological methods has not enabled us to detect any morphological features in the nuclei that belong to this "sesquiphasic" size and would allow us to interpret the real significance of this intermediate step. At present we can only infer the existence of this phase by means of statistical analysis of the volumes of interphasic growth. More suitable material would perhaps reveal some morphological detail that would be useful. In the discussion of results, we shall return to the problem of the nature of the sesquiphasic step, which has been discussed in our previous papers (Schreiber, 1943, 1946, 1947).

2. (*d*) *The caryometric analysis of uterine tissues during the sexual cycle*

As explained before, we have tried to analyze interphasic growth of nuclei in tissues where a specific morphogenetic stimulation was obtained with a suitable dose of hormones. Cutting off the supply of these hormones (as in castration), and followed by intensive treatment with hormones of the gland that had been removed, enabled us to study nuclear growth and to arrest it at both ends of the growth cycle.

The uterus in mammals during the normal estral cycle and pregnancy provides a medium for studying the same process of synchronous cell proliferation under physiological conditions, and for comparison with the above mentioned experimental conditions (castration and estrogenic treatment of castrated animals).

In the course of our work, facts have appeared which facilitate the statistical study of interphasic growth and give us the full picture of its rhythm. Many problems in endocrinology arise and these are treated separately (Salvatore, 1948, a, b, c). We have limited ourselves here to analyzing the statistical and cytological side of the phenomena, correlating this with the results obtained in other fields in which interphasic growth has been studied (Schreiber, 1943, 1946 a, b, and c, and 1948).

The studies reported here were performed by measuring nuclear volume and analyzing its statistical variability using the same general methods as those mentioned above. The first layer of the uterine epithelium, the glandular cells, and the muscular fiber of the meiometrium were examined during the estral cycle and pregmancy in white rats, mice and humans. In the castrated animal, we studied nuclear volumes in the untreated female, and during experimental estrus induced by injection of estrogenics.

In all cases the nucleus was considered as a rotating ellipsoid, and only those parts of the tissue with the nuclei well oriented for measurement were studied. We give here some typical cases representing respectively an estral cycle, a castration,

an experimentally induced estrus, and a pregnancy, which give us the clearest picture of rhythmic nuclear variation. A complete description of all cases, with histograms and numerical tables, is recorded in the papers by Salvatore and Schreiber (1947), Salvatore (1947–48), and Schreiber, Mello and Salvatore (1949).

The following facts were observed: During the period of diestrus all the nuclei are simultaneously at rest, at a basic volume which we conventionally call "1." During the period of increase in hormonic activity, the nuclei begin to grow, showing a rhythmic pattern (polymodal frequency curves) reaching double size after having stopped temporarily at the intermediate stage of 1.5 times initial size. The phenomenon is exactly the same in all categories of cells studied, and we believe that muscle cells are of special interest, since their cyclic growth has hitherto been completely unknown.

When the hormone reaches its maximum of concentration at estrus, the nuclei seem to stop simultaneously after reaching size 2, as though they were waiting for some new conditions which would allow them to begin mitosis. Some nuclei undergo further volumetric rhythmic growth and reach sizes 3 and 4.

During estrus and the succeeding short transitional stage (estrus-metaestrus) mitosis appears in many cells by a change in the hormonic conditions (the nature of which is still under discussion by physiologists, i.e. quantitative or qualitative), and statistical analysis of the nuclear sizes reveals the reappearance of lower volume categories. Some cells degenerate and are probably phagocytized; others begin a new growth cycle. At the end of this period, no mitosis is present (metestrus) and all the nuclei are once more resting at size 1, just as they were during the diestrus stage at which the cycle was begun (Fig. 2).

The morphological features of the nuclei during the phases of the cycle are slightly different. The nuclei of the initial stages are more likely stained and of more compact aspect than those of double size, which have one or two clearly visible nucleoli. These facts appear in both epithelial and muscular cells. An interesting modification in the morphological aspect of the nuclei has been reported by Pfeiffer and Hooker (1944) in the stromal tissue of the uterus of mice under different hormonic conditions, some of which can be compared with ours. It does not seem impossible that the features described by these authors belong to the phases of a typical "endomitotic cycle" (endoprophase, endometaphase, etc.), but the authors do not attempt any interpretation and do not give any detailed statistical record of the volumes.

During pregnancy there is a situation identical to that of the estrus stage, complicated in the rat by an intermediate cycle of cell division on the 13th and 14th days. Of special interest was the muscular layer, in which we found a number of cells continuing rhythmic growth and reaching high multiples of the basic volume. This condition is identical in the uterine segment bearing the foetus which is mechanically enlarged, as well as in the intermediate segment without foetus. This eliminated the idea of hypertrophy, of a mechanical origin, of the muscle cells, which some authors believe.

These studies elucidate from a quantitative point of view, the nature of uterine hypertrophy and hyperplasy. Rhythmic growth of the nucleus appears to provide a new explanation for the hypertrophy of cells during pregnancy and estrus growth, i.e., as being due to interphasic growth and subsequent division (hyperplasy), or

interphasic growth without subsequent division but followed by many successive endomitotic cycles (true hypertrophy) (Salvatore, 1948 c).

In the untreated castrated animal the caryometric picture is exactly the same as that during diestrus and metaestrus; i.e., an absolute rest of all the nuclei at the basic volume 1. The experimentally induced estrus and the successive estrus-

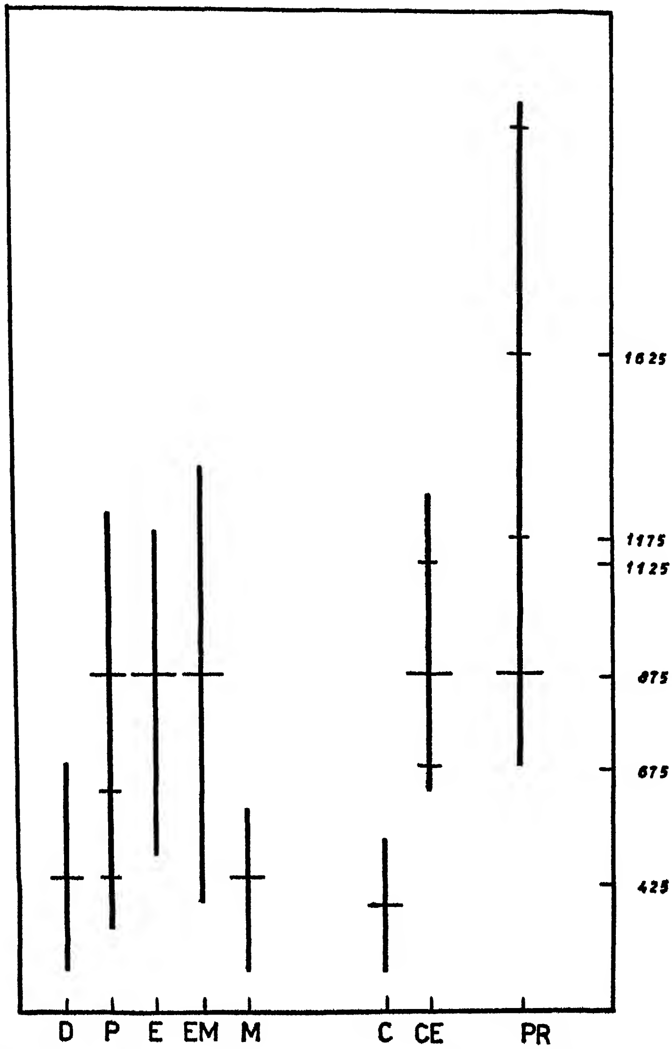

FIGURE 2. Nuclear sizes of rat miometrium during the stages of the estral cycle, castration, experimentally induced estrus, and pregnancy.

The vertical lines represent the total range of variation of nuclear volumes. The longer transverse lines represent the main modal values; shorter transverse lines represent the secondary modes. Values on the right side of the diagram give the average of the modal values of all stages. D = diestrus; P = proestrus; E = estrus; EM = estrus-metestrus; M = metestrus; C = castrate; CE = experimental estrus in castrated animal; PR = pregnancy.

metaestrus stage obtained by the interruption of the hormone supply are identical, however, and clearer than the corresponding physiological stages.

We can infer from the above facts that the increase in hormonic concentration results in the volumetric increase of the uterine cell nuclei, and this fact manifests itself by a doubling of the volume one or more times. During physiological estrus and during the interruption of the hormonic treatment, mitotic activity appears, and the factors which induce mitosis find practically all the nuclei ready to begin the prophase and divide ("mitosebereit" of Hertwig).

The volume of the prophase (size 2) and that of the resting nuclei in castrated animals (size 1) give us the extremes of a duplicating cycle, just as the volume of the meiocyte gave the limits of interphasic growth of the spermatogonia.

This indicates the true interphasic nature of nuclear growth during the physiological phases of the uterus and the action of hormone stimulation, and can hardly be explained by a simple water imbibition or some colloidal modification of the cell, as some authors believe.

Comparing the total range of the histogram of the nuclear volume of the castrated rat and that of the rats experimentally maintained in estrus (Fig. 2), it appears evident, from the lack of any overlapping of the histograms, that there are not two or more categories of cells of different specific initial sizes, originally present in the tissue, as some authors believe; the statistically recorded modifications consist of a real interphasic growth of all or nearly all the cells simultaneously in the tissue.

In the uterine cell it is therefore possible to halt interphasic growth by means of specific endocrine conditions at both ends of the cycle. The intermediate steps during reduplication (sesquiphase) are revealed by the frequency curves with unusual clarity, and from the total absence of intermediate size classes in the castrated animals, we can infer that this sesquiphase is also a real growth phase and not a different category of cell size.

2. (*e*) *The caryometric analysis of ovarian endocrine tissues*

During the development of the graffian follicle, the cells of the granulosa layer undergo repeated mitotic divisions. We thus have here another homogeneous tissue in active multiplication, and we can apply the same general principle of the statistical analysis of interphasic growth.

After the bursting of the follicle, the transformation of the follicular cells is accomplished by enlargement of the nucleus and the cytoplasm. The statistical analysis of this phase gives us another clear picture of the rhythmic growth of the nucleus.

Figure 3 represents the modal values and the total range of variation of nuclear size in a developing follicle and in the corpus luteum. At the beginning of the follicle development, the oocyte is surrounded by only one layer of cells. The nuclei of follicular cells at that stage are predominantly at basic volume and some nuclei are at 1.5 times greater. In a more developed follicle the histogram shows three distinct modes: volumes 1, 1.5, 2 respectively. The prophases are all at size 2. We have here the same condition as previously stated in the uterus layers and in the spermatogonia. The growth cycle of the cell consists of a duplication of the nuclear volume showing the intermediate step (sesquiphase) and ending with the prophase.

The granulosa cells have a mitotic index of about 10 per cent during the period

of the growth of the follicle, which drops to 0 per cent at the moment of luteal transformation.

The luteal cell of a transitory corpus luteum has a very regular statistical distribution of nuclear volumes with only one mode.

This mode corresponds exactly to the volume of the prophase (size 2) of the granulosa cells of the follicle. In the corpus luteum during pregnancy there is a further volumetric growth of some cells whose nuclei reach size 3, and probably 4 and 6.

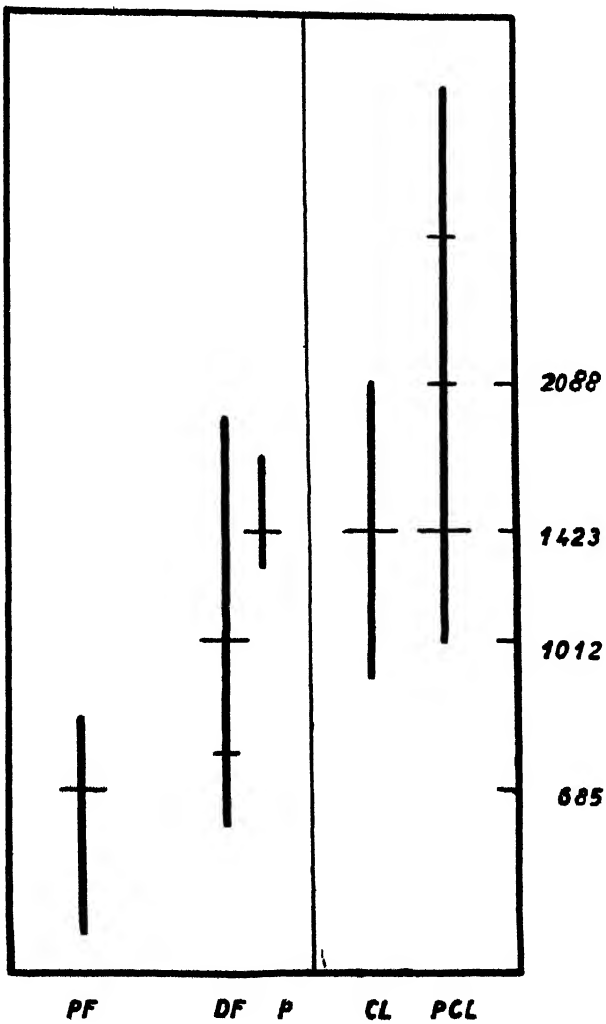

FIGURE 3. Nuclear sizes of the cells of graffian follicle and corpus luteus in the rat. Same explanation as Figure 2. PF = primary follicle; DF = developing follicle; P = prophases of developing follicle cells; CL = corpus luteus (cyclic); PCL = corpus luteus in pregnancy.

These higher multiple values of the phasic one are more difficult to establish owing to their rarity and the greater variability of the larger nuclei. There are no apparent differences in the morphology of these different classes of nuclei.

It is generally assumed that the luteal cells originate from the granulosa cells, although some authors believe that the cells of the "theca interna" of the follicle also contribute to their formation. The nuclear sizes of the cells of the granulosa layer and those of the luteal cells form a series of rhythmic values (Table II). This indicates the probable origin of the luteal cells from the granulosa; these reach the end of interphasic growth, and instead of beginning the prophase and dividing, continue the endomitotic growth and become transformed into the luteal cells under the influence of the proper hypophyseal hormone. These luteal cells can grow still further, with endomitotic cycles reaching rhythmic values higher than those of the initial size.

We must here recall the discussion on use of the term "endomitosis" in a broad sense, meaning reduplication of the nuclear genes without nuclear division. This reduplication may be accompanied by a reduplication of the number of chromosomes (true polyploids) or by that of the chromonemata within the chromosomes without variation of the original ploidy (polytenic chromosomes). Furthermore this reduplication of the nuclear content may be accomplished in some cases by the appearance of the morphological stages of Geitler's "endoprophase," "endometaphase," "endoanaphase" and "endotelophase," or in other cases without any morphological manifestation of the chromosome nucleinization cycle [as in the case of the ileum cells of the mosquito (Berger and co-workers)].

The luteal cells belong to the last category of cells, showing no visible variation of the inner feature of the nucleus in the different classes of size.

We should perhaps relate these results to Painter's studies on nuclear participation in the secretory cycle of cells (Painter, 1945). The increased need for cytoplasm ribosenucleic acid in the actively functioning cell is, as Painter thought, supplied by a reduplication of the nuclear genome and is manifested in the different types of glands by successive mitotic cycles or by endomitotic growth of the nucleus. We could perhaps raise the question as to whether this nuclear activity during the secretive processes might also explain the interphasic nature of the observed phenomena in such cells as the follicular and luteal, in which the chemical constitution of the secretion is not, at least, in the ultimate stage of proteic nature.

Summarizing the results of the statistical study of ovarian cells we can furthermore emphasize the fact that the rhythmic growth of the nucleus represents true interphasic growth, having an intermediate step at 1.5 times the initial size of each duplicating cycle.

3. Discussion and Conclusion

Before trying to describe the quantitative characteristics of nuclear growth during interphase, we must bear in mind that during this period, caryometric analysis can give only a rough, quantitative aspect of what occurs in the nucleus, and only by comparing the steps reached under different conditions and using material in which the internal characteristics can be studied can we try to draw some definitive conclusions.

It should not be forgotten that nuclear size is the result of a number of physical, physico-chemical, and chemical phenomena acting during the period in which the genes reduplicate and probably during which they perform their specific action in

the cells. It is not definitely ascertained at what moment of the nuclear cycle reduplication takes place, although the interphase seems to be the most probable. We cannot affirm either whether gene reduplication and chromosome splitting are simultaneous, because they occur at different levels of molecular and morphological organization. Furthermore, we must limit our analysis to the simplest case of the interphase of somatic cells, and not extend it to the most complicated cases of auxocytic growth, post-meiotic divisions or segmentation of the blastomeres, in which other conditions (i.e., multiple strand constitution of the chromosomes) would complicate the analysis.

Caryometric studies with statistical analysis of the prepared tissue conducted at the same time as the motion pictures of the living nucleus (Wermel and Portugalow, 1935) confirm that rhythmic growth, as deduced from the modal values of the frequency curves of nuclear volume, corresponds to a real discontinuous growth. The modal values correspond to the steps reached after each growth period is ended. The studies conducted in the regenerating liver by use of colchicine methods (D'Ancona, 1939–41–42) and in the neoplastic tissues by statistical methods (Biesele, Poyner and Painter, 1941) confirm the close correlation between multiple modal values and polyploid or politenic status of the chromosome complement.

The experiments we have carried out in the spermatogenetic stages and in the uterine and ovarian cells might give a more complete picture of rhythmic growth, because of material and physiological conditions that permit the recognition of the true interphasic nature of that discontinuous growth.

We can summarize the facts as follows:

(1) Comparison of the volumes attained at the successive steps of the spermatogonium mitotic cycle, with the volumes at the stages of the meiotic elements (in the vertebrate testicle), allow us to measure variations in volume in terms of quantitative values of the genome.

(2) During the mitotic cycle the prophase represents the end of a growth cycle of the nucleus and corresponds physiologically to the completion of a reduplication cycle of the nuclear genomes. In all cases the volume attained during prophase corresponds to volume 2 of the rhythmic growth series, thus indicating the interphasic nature of the rhythmic steps.

(3) Using the physiological conditions of the sexual cycle in mammals we can study interphasic growth in the nuclei of a specific tissue sensitive to the stimulus of corresponding specific endocrine conditions. These conditions permit growth to be stopped at both extremes of the nuclear cycle. Here too the prophasic volume gives us the limits of a reduplication cycle of the chromosomes, and allows us to consider rhythmic growth as truly interphasic.

(4) The whole series of rhythmic values of nuclear size in all the tissues studied indicates that this growth is accomplished by a succession of reduplicating cycles, but is complicated by the existence in each reduplicating cycle of an intermediate phase ("Zwischenklasse") in which the nuclear volume is one and a half times the initial volume of each cycle ("Sesquiphase").

The ratios between the steps thus appear to be alternately 1 : 1.5 and 1 : 1.33, and the whole series of values should be 1 : (1.5) : 2 : (3) : 4 : (6) : 8 : (12) : 16 etc., the steps in parenthesis being the so-called sesquiphases.

These facts clearly appear from the scatter diagram of Figure 4. Table II shows the regression of the modal volumes in the theoretical rhythmic growth series with

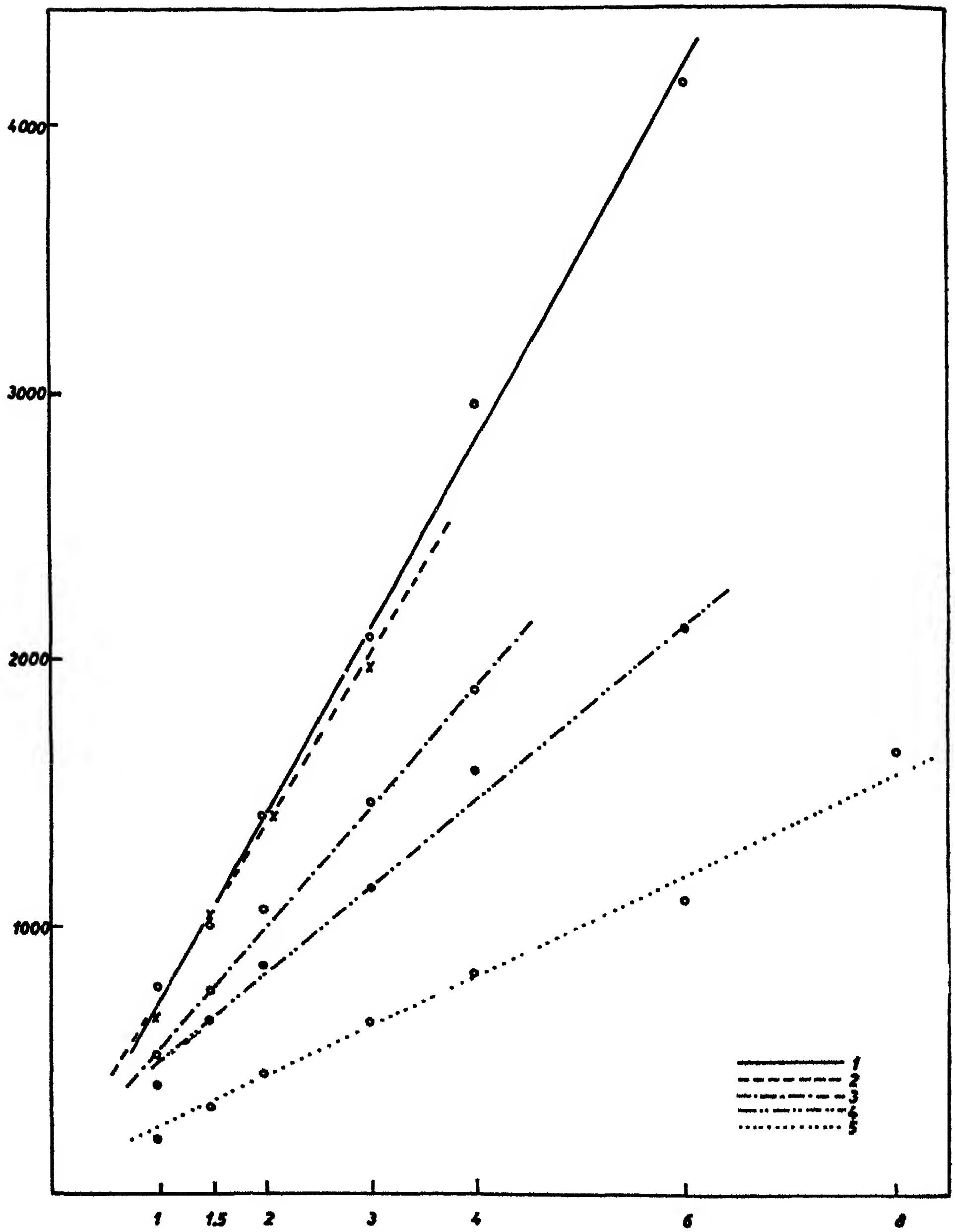

FIGURE 4. Scatter diagram and regression lines between modal values of nuclear volumes of uterine and ovarian cells and the theoretical series of the rhythmic growth stages with the intermediate step (sesquiphase).

(1) Graafian follicle and luteal cells in the rat. (2) Endometrium of the rat. (3) Human miometrium. (4) Miometrium of the rat. (5) Miometrium of the mouse.

the intermediate steps of the sesquiphase. We cannot discuss here the problem of th absolute values of nuclear volume in various tissues of an organism, which was considered in the original research of Jacobj, but which we believe should be further analyzed.

Rhythmic growth of the cell nucleus thus appears to be related to interphase activity. The size of the nucleus increases with a period of active growth, alternated with periods of rest. After reduplication of genic material is accomplished, generally the nucleus begins the prophase stage and divides. Sometimes mitosis is suppressed and a new reduplicating cycle (endomitosis) begins.

From the relationships between modal volumes and the multiple value of the chromosomes or chromonemata we must infer that at each step after a growth cycle, the nucleus consists of the genes and the material accompanying them which form the chromosomes, the nucleolus, and nuclear sap, the quantity and physico-

TABLE II

Nuclear volume (modal values) of the uterine and ovarian tissues (P = prophase)

	Series of modal values of the rhythmic growth of the nucleus						
Theoretical series	1	1.5	2	3	4	6	8
Rat endometrium	665	1037	1460 1428 P.	2100 1983 P.			
Rat follicle and luthcal cell	685	1013	1423 1436 P.	2088	2961	4157	
Rat miometrium	420	651	863	1152	1592	2108	
Mice miometrium	216	337	444	642	830	1100	1650
Human miometrium	522	767	1078	1473	1891		

chemical status of which determine, in regularly shaped nuclei, a nuclear size proportional to the number of genomes present in the nucleus.

This principle makes it possible for us to understand the whole mass of facts revealed by caryometric analysis. We must consider as a fundamental fact the constancy of the ratio between the genes and the accessory materials that accompany them in the morphological constitution of the nucleus, both in quantity and in physico-chemical status, during the true "resting" condition between two successive reduplication cycles.

During the "metabolic" period (interphasic growth period) this ratio is obviously altered by the phenomena of water and material changes between the nucleus and the cytoplasm; but the ratio goes back to its initial value at the end of each cycle.

The nature of the intermediate step during the reduplication cycle, which we call "sesquiphase," is more difficult to understand. We do not know of any cytological feature of the nucleus specific for that phase; its evistence is only inferred from statistical analysis. We note that these steps do not appear in all tissues or in all

species. The classical series of Jacobj and of many others give us a clear picture of a purely reduplication series.

Notwithstanding, in many other cases recorded by previous authors and in the ones here studied, as well as in the nuclear diminution series studied by Schreiber and Romano-Schreiber (1941) the existence of these intermediate steps is evident. It is not possible to call upon factors involving the geometric form of the nucleus in determining those steps, because they appear in both perfectly spherical nuclei (spermatogonium and luteal cell) and elliptical ones (endometrium and miometrium).

If we compare the histograms of the same tissue under different physiological conditions, we see that the same modal value always appears at the same position, and often what is the main mode in one physiological condition can be a secondary modal value in another. This fact allows us to consider the "sesquiphase," which statistics indicate to be a true biological phenomenon.

The theoretical explanation of the sesquiphase may be attempted by different methods and we can try to analyze some of them here. We might first look for a physiological explanation as G. Hertwig and Brummelkamp did. We can imagine some reasons for a stoppage or slowing down of the growth when approximately 50 per cent of the initial volume is attained. We cannot analyze here the complicated and perhaps artificial theories of G. Hertwig, based upon a hypothetical factor acting in different categories of nuclei, and correlating the chromosome number in some cases with the nuclear volume and in other cases with the nuclear surface. The mathematical explanations of Brummelkamp appear even more fantastic. Both authors consider the ratio between the steps as being 1:1.41, i.e., the square root of 2, which is very close to the ratio 1:1.5 considered correct by other authors. Wermel and his co-workers also consider the ratio to be 1:1.5. According to these authors the series should be 1:1.5:2:2.25. . . . For that reason these authors believe that the "Verdoppelungsgesätz" of Haidenhain and Jacobj must be rejected, but they do not give any new explanation of the nature of the rhythmic step of the growing nucleus.

We might also try to explain the "sesquiphase" in some other way, for example, by relating a different velocity of the splitting of euchromatic and heterochromatic regions of the chromosomes. Or we can take into consideration the different effects upon nuclear and nucleolar size of the two different types of chromatin (Fernandes and Serra, 1944). All this, however, would not explain the constancy of the ratio 1:1.5 between the steps in many categories of cells in which the sesequiphase can be detected, and which have a great variety of ratios of metero- and euchromatin.

Here it is interesting to note several facts found by Biesele (1940) relating to the 50 per cent increase in volume of the metaphasic chromosomes under certain physiological conditions without an apparent increase in the number of chromonemata. We cannot at present imagine what relation this may eventually have to the phenomena analyzed by us here, but we presume that these phenomena may eventually be taken into consideration.

Many other explanations could be offered in more purely speculative fields, such as for instance, different mathematical laws relating nuclear volume to the chromosome content in the different periods of the interphase, but here too, the constancy of the 1:1.5 ratio limits the possibilities of the hypothesis.

From a more genetic point of view, Heidenhain, since the very early studies of Jacobj, admitted that the intermediate values which in some cases appear as an

exception to the "Verdoppelungsgesätz" could be explained by admitting that the two halves of the nuclear content derived respectively from maternal and paternal origin, reduplicate independently (Jacobj, 1931).

In the case in which only one reduplicates, the duplication ratio is not maintained. Hertwig (1937) admitted a similar point of view but subsequently rejected it without any justification, preferring the above mentioned theory of the two different factors acting on the surface or on the volume of the nucleus.

On the basis of the facts analyzed in this paper we can perhaps try to support more clearly this genetic point of view. We can admit (Schreiber, 1943) that in a diploid nucleus each chromosome set of different gametic origin represents a physiological entity during the reduplication process, and one set may be more precocious than another.

The influence of gametic origin on the behavior of an entire set or on some special chromosomes also manifests itself in other phenomena of the cell cycle (White, Schrader, etc.): for instance, the precocious condensation of one haploid set in the scale insect, or the elimination of the paternal chromosomes in Sclara. A difference in the initial rhythm of reduplication between maternal and paternal chromosomes of each original pair is also invoked by Holt (1917) to explain the existence in Culex of the "six series" or "nine series" of chromosome numbers, in intestinal cells.

We could thus represent in an hypothetical way the so-called "sesquiphase" as a transitory stage in which one haploid set (or its multiples) has reduplicated and the other has not yet done so. The "quantum" of the simultaneous reduplication of the genes would in that case be the haploid set, or "genome."

All this is merely speculation which might perhaps lead to new research in a purely cytological field. As stated above we have at present no cytological evidence of the sesquiphase stage, which can only be detected by the statistical analysis of the growing nucleus.

As a general conclusion we can state that caryometric methods, when strengthened by the physiological conditions which specifically influence interphasic growth and mitosis, can help the cytologist to make a closer study of the growing nucleus and to formulate some suggestions on its quantitative aspect and on the dynamics of gene reproduction.

4. Summary

The author analyzes present knowledge on the problem of "rhythmic growth" of the nucleus as it appears from the point of view of statistical caryometric research. This analysis is carried out especially with regard to the problem of the intermediate steps during reduplication of nuclear volume to about 1.5 times the initial size.

The nature of these steps is analyzed experimentally in three different fields: (1) Interphasic growth of spermatogonia, whose nuclear size is compared with a meiocyte with a known number of chromosomes. (2) Interphasic growth of the uterine cells under different physiological conditions. (3) Interphasic growth of the granulosa cells of the ovary, and the transformation into luteal cells.

The interphasic nature of rhythmic growth is considered also as a possible explanation for the intermediate step during reduplication of nuclear size that is called sesquiphase.

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HOPKINSIAXANTHIN, A XANTHOPHYLL OF THE SEA SLUG HOPKINSIA ROSACEA

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Carotenoid pigments of land animals are usually one or more of the common, yellow constituents of their vegetable food (Zechmeister, 1934). By contrast, carotenoid pigments of marine animals, especially invertebrates, are seldom identical with the yellow constituents of marine plants (Fox, 1947; Lederer, 1940).

Another example of a carotenoid pigment found thus far only in a marine animal is the unusual rose-pink coloring matter of the nudibranch mollusk *Hopkinsia rosacea*. Hopkinsia is found in such small numbers along the coast of California that only one or two organisms, weighing but a gram or two, have been available at any one time. As a result, studies of the extracted pigment have been restricted to determinations of optical properties such as the characteristic spectral absorption curves, relative adsorbability in Tswett adsorption columns, phasic behavior and color reactions. These properties provide a basis for some deductions regarding the molecular structure of the Hopkinsia pigment. They indicate that this substance, which has not been described before, is a xanthophyll-like carotenoid. In accordance with widely accepted carotenoid nomenclature, this molluscan pigment is called hopkinsiaxanthin.

Experimental

Hopkinsia rosacea was collected at low tide at Moss Beach, San Mateo County, and at the Monterey Peninsula, Monterey County, California. When removed from its habitat, this organism proved to be exceptionally delicate and fragile; hence, specimens were brought to the laboratory in moist seaweed and placed immediately in about 100 ml. of methanol or ethanol. These alcohols removed all the pigment and yielded orange-yellow solutions. Pigments in these alcoholic extracts were transferred to petroleum ether. To this end, about 50 ml. of petroleum ether (B. P. 50–70°) and a large volume of water or of salt solution were added to the alcoholic solutions. When dissolved in petroleum ether, the extracted pigments formed a yellow solution in contrast to the orange-yellow color of the alcohol solution.

Adsorption of the petroleum ether solution of the extracted pigments on a column of powdered sugar (2.4 by 10 cm.) yielded a red-orange zone near the top of the adsorbent. When the adsorbed pigments were washed with petroleum ether plus 0.25 per cent n-propanol, most of the coloring matter moved rapidly through the column as a red-orange zone which contained the hopkinsiaxanthin. The colorless percolate below this red-orange zone indicated that carotenes and esters of hydroxy carotenoids were absent, because most of these substances are not adsorbed on columns of sugar. Above the red-orange hopkinsiaxanthin in the Tswett column,

there appeared about five pale, indistinct, red-orange and yellow zones which were not examined further.

Hopkinsiaxanthin, contained in the principal red-orange zone in the adsorption column, was recovered by removal of the adsorbent with a spatula followed by elution of the pigment with alcohol. Readsorption of the pigment on columns of powdered sugar, of Celite and of activated magnesia always yielded a single band. These results indicate that a single pigment had been isolated (Strain, 1942, 1948). This same pigment was obtained from organisms collected in 1943, 1946, and 1947.

The physical and chemical properties of hopkinsiaxanthin are similar to those of the carotenoid pigments, particularly the keto carotenoids. As with the ketonic carotenoids, the color of solutions of hopkinsiaxanthin varies with the solvent. At

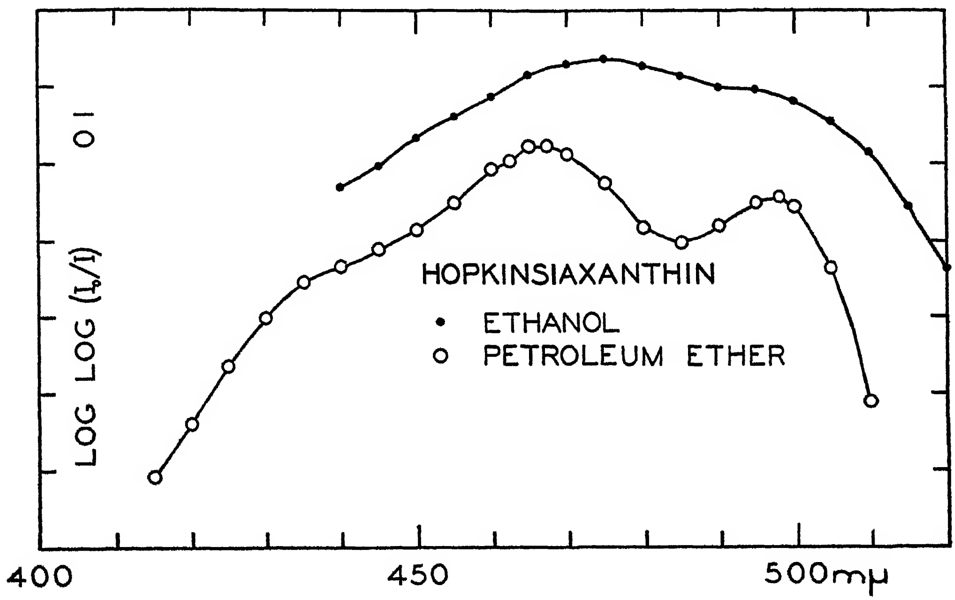

FIGURE 1. Characteristic spectral absorption curves of hopkinsiaxanthin measured in petroleum ether and in 95 per cent ethanol.

equal concentration of pigment, solutions of hopkinsiaxanthin in nonpolar hydrocarbons are a lighter yellow than solutions in polar solvents such as alcohols. This effect, which is readily reversible with change of solvent, is illustrated by the spectral absorption curves reproduced in Figure 1.

As shown by partition experiments, hopkinsiaxanthin is very much more soluble in 90 per cent methanol than in petroleum ether. With 80 per cent methanol, some of the xanthophyll dissolves in the petroleum ether, but most of it remains in the alcohol layer. With 70 per cent methanol, most of the pigment dissolves in the petroleum ether layer.

The adsorbability of hopkinsiaxanthin varies with the adsorbent and the solvent. This xanthophyll is strongly adsorbed on sugar or on Celite when petroleum ether is used as the solvent. It is but weakly adsorbed on these adsorbents when alcohol,

petroleum ether plus alcohol or petroleum ether plus acetone are used as solvents. From these polar solvents the hopkinsiaxanthin is so strongly adsorbed on activated magnesia that acids must be added in order to elute the pigment. On powdered sugar and on Celite, the adsorbed pigment usually appears red-orange, but at low concentration it is salmon colored. Adsorbed on magnesia, the pigment is red-orange even when adsorbed at low concentration.

Adsorbability of hopkinsiaxanthin relative to some common plant carotenoids and chlorophylls also varies with the solvent as is illustrated in Table I. In columns of activated magnesia (plus siliceous filter aid), hopkinsiaxanthin is more tenaciously adsorbed than all the common xanthophylls including the very strongly adsorbed rhodoxanthin. Indeed, it is so strongly adsorbed that it cannot be washed along in the adsorption columns with the strongly polar solvents ethanol and acetone.. In this respect, hopkinsiaxanthin resembles the diketonic carotenoid rhodoxanthin, which is strongly adsorbed on columns of magnesia and but weakly adsorbed on columns of powdered sugar or of Celite (Strain, 1948).

TABLE I

Effect of different solvents upon the adsorbability of hopkinsiaxanthin relative to chlorophylls a and b, lutein and cryptoxanthin in columns of powdered sugar

Petroleum ether	Petroleum ether + 5 per cent acetone	Petroleum ether + 0.25 per cent *n*-propanol
Chlorophyll *b*	Chlorophyll *b*	Chlorophyll *b*
Chlorophyll *a*	Chlorophyll *a*	Hopkinsiaxanthin + Lutein
Hopkinsiaxanthin	Hopkinsiaxanthin	Chlorophyll *a*
Lutein	Lutein	Cryptoxanthin
Cryptoxanthin	Cryptoxanthin	

Hopkinsiaxanthin exhibits many color reactions that are characteristic of the carotenoid pigments. A solution of the Hopkinsia xanthophyll in diethyl ether shaken with concentrated hydrochloric acid yields a clear blue color in the acid layer, the blue color remaining unchanged for at least one-half hour. A petroleum ether solution treated with concentrated hydrochloric acid fades rapidly and forms a blue precipitate at the interface between the two liquids. A chloroform solution reacts with antimony trichloride yielding a blue color that also fades rapidly. Strong phosphoric acid (85 per cent) extracts all the hopkinsiaxanthin from petroleum ether and yields a blue color in the acid layer.

Strong alkalies convert hopkinsiaxanthin into a pale yellow pigment. For example, when a solution of this xanthophyll in petroleum ether is shaken with a 10 per cent solution of potassium hydroxide in water, the color changes from deep yellow to light yellow, although most of the pigment remains in the petroleum ether. When this petroleum ether solution is adsorbed on sugar, a weakly adsorbed, yellow band is formed. This yellow band is less adsorbed than the original, unaltered xanthophyll. Solutions of potassium hydroxide in methanol extract the hopkinsiaxanthin from the petroleum ether and the pigment fades rapidly.

Hopkinsiaxanthin dissolved in petroleum ether is but slightly affected by traces of iodine plus dimethylaniline (Strain, 1941). This reaction yields small amounts of pigments that are more strongly adsorbed than the unaltered xanthophyll on columns of powdered sugar.

Discussion

The characteristic color reactions of hopkinsiaxanthin and the effect of polar and nonpolar solvents upon the spectral absorption properties (Fig. 1) suggest that this pigment may be a ketonic carotenoid. The weak adsorbability of hopkinsiaxanthin on columns of powdered sugar and the pronounced adsorbability on columns of magnesia (Table 1) also support this conclusion.

As indicated by the wavelengths of the absorption bands, the hopkinsiaxanthin molecule contains about 11 double bonds with at least one of these in the form of a carbonyl group. The stability of the pigment toward solutions of iodine suggests that all these double bonds occur in the more stable, trans, spatial arrangement rather than in labile, *cis* configurations.

Because the adsorbability of hopkinsiaxanthin on columns of powdered sugar approximates that of lutein (dihydroxy alpha-carotene), it is possible that thé molecule contains one or two hydroxyl groups. The preferential solubility of hopkinsiaxanthin in methanol relative to petroleum ether suggests that there are probably no esterified hydroxyl groups.

With respect to solubility and color reactions, hopkinsiaxanthin resembles only one of the principal carotenoid pigments isolated from marine plants; namely, fucoxanthin. However, the Hopkinsia pigment is not identical with fucoxanthin as shown by the wavelengths of the absorption bands: 466 and 497 mμ for hopkinsiaxanthin in petroleum ether (Fig. 1) and 449 and 477 mμ for fucoxanthin in petroleum ether (Strain, Manning and Hardin, 1944). It also differs from fucoxanthin with respect to adsorbability, for it is more strongly adsorbed than fucoxanthin on columns of magnesia, and it is less strongly adsorbed than fucoxanthin on columns of powdered sugar.

Summary

The striking, rose-pink color of *Hopkinsia rosacea* is due to the presence of a carotenoid pigment, hopkinsiaxanthin. This xanthophyll, which has not been found in plants or in other animals, occurs in the stable, trans configuration and probably contains a carbonyl group.

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ANDROGENESIS, A DIFFERENTIATOR OF CYTOPLASMIC INJURY INDUCED BY X-RAYS IN HABROBRACON EGGS[1]

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Introduction

Any technic which differentiates cytoplasmic from chromosomal injury induced in the living cell by x-rays is of interest to investigators of biological effects of ionizing radiations. Injury to chromosomes is measured by their breakage and rearrangement and by visible and lethal mutations. Injury induced in the cytoplasm must, ordinarily, be studied by means of tests for physical and chemical changes in treated cells or by changes in behavior of the cell as a whole, inhibition of division, etc.

When evidence for the nature of effect is incomplete, it is always tempting to attribute greater sensitivity of egg than of sperm to cytoplasmic injury because of the extreme difference between these two types of cells in respect to amount of cytoplasm. Muller (1937) writes, "In Drosophila, a given irradiation of the eggs shortly before or after fertilization will result in non-development and death of a high proportion of them, whereas the same amount of treatment of spermatozoa alone allows more of the fertilized eggs to develop. Nevertheless genetic analysis of the resulting adults proves that they contain more genetic changes, *and more drastic ones,* in the latter case than in the former. The *difference* in death rate here, then, must have been of non-genetic origin, involving, no doubt, some injurious change in the egg protoplasm. On the other hand, the death of the embryos that were derived from treated sperm and untreated eggs must have been genetic." In experiments on irradiation of females of Drosophila, dose has not exceeded 6000 r.

Whiting, P. W. (1938) found that some eggs of Habrobracon survived a dose of 18,000 r (lethal dose for sperm in this species is about 10,000 r) and Whiting (1938) identified these as eggs irradiated in first meiotic prophase. Eggs treated in first meiotic metaphase were much more sensitive than sperm. This order in respect to size of lethal dose also holds for Sciara as reported by Metz and Boche (1939) and Reynolds (1941). In contrast to the results cited by Muller, then, eggs of Habrobracon and of Sciara are either more sensitive than sperm or less so according to condition of the chromosomes at time of treatment.

It is a generally accepted fact that the stage in the nuclear cycle is of importance in determining degree of sensitivity to x-rays and Muller in his epoch-making paper on artificial transmutation of the gene (1927) writes, "In addition, it was also pos-

[1] This investigation was completed with the aid of a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service. The work was done at the Zoological Laboratory of the University of Pennsylvania and at the Marine Biological Laboratory, Woods Hole, Massachusetts. The author is grateful to these institutions for the use of laboratory facilities and to Mr. L. R Hyde for administering the x-ray treatments.

sible to obtain evidence in these experiments for the first time, of the occurrence of dominant lethal genetic changes, both in the X and in the other chromosomes. Since the zygotes receiving these never develop to maturity such lethals could not be detected individually but their number was so great that through egg counts and effects on the sex ratio evidence could be obtained of them en masse." This refers to dominant lethal changes induced in the sperm.

There is no argument, *a priori,* for the supposition that sperm have a monopoly on dominant lethal chromosome effects. The treatment of eggs in a nuclear stage which responds to relatively low doses of x-rays by the production of dominant lethal genetic changes will explain the greater sensitivity of eggs than of sperm.

The "genetic analysis of the resulting adults" to which Muller refers is an analysis of recessive or of non-lethal changes. Dominant lethal genetic or cytological effects are, of necessity, "strained out" in the process of reaching adulthood. As Sparrow point outs (in press), "In the presence of a low frequency of rejoining, an increased percentage of acentric fragments or deletions would be expected and thus a higher proportion of lethality would occur. Paradoxically, therefore, if one were scoring for aberrations in the F_1 generation, following radiation of one or both of the parental gonads, the recovered aberrations would not necessarily represent a true picture of total chromosome breakage since cells in the most sensitive stages would be the least likely to produce viable F_1's."

Habrobracon eggs vary greatly in their response to x-rays according to stage at time of treatment, and in the most sensitive stage studied (four times as sensitive as the sperm), cytological observation of large numbers of them after treatment has shown that death is due to chromosomal injury. The chromosomes of Habrobracon ($2n = 20$) are small, however, and any evidence for these conclusions gained from a different method of approach is of value and should help to convince those accustomed to work with large chromosomes who tend to be skeptical of observations made on small ones.

Androgenetic males, developed from the untreated sperm nucleus in x-rayed cytoplasm, furnish material for this different method of approach. A perfectly normal, fully fertile individual must, of necessity, have developed in an egg with cytoplasm not seriously altered by its exposure to x-rays.

Material and Methods

Wild type stock (No. 33) of the parasitic wasp, *Habrobracon juglandis,* with a hatchability of 96–98 per cent, has been used for all experiments since the beginning of this study in 1937.

Homozygous females of this stock were x-rayed and mated to untreated males with one or more traits recessive to wild type. These females were placed with host caterpillars, and their eggs were studied in respect to hatchability and/or were allowed to mature. Viable unfertilized eggs develop into wild type, gynogenetic males (both chromosomes and cytoplasm irradiated). Viable fertilized eggs develop into wild type, heterozygous, biparental, diploid females (chromosomes half irradiated, half not, cytoplasm irradiated). Fertilized eggs most seriously injured in respect to egg chromosomes develop into recessive, androgenetic, haploid males (chromosomes not x-rayed, cytoplasm x-rayed) (Whiting, 1946a). The present

paper is concerned primarily with the two types of males. X-ray induced changes common to both afford proof for cytoplasmic injury.

Eggs were separated according to time of laying into those irradiated in first meiotic metaphase (metaphase I) and those treated in first meiotic prophase (prophase I) (Whiting, 1945a).

For x-ray treatments a dual-tube self-rectifying outfit with a simultaneous cross-firing technic was used. The secondary voltage was 182 kv. and the tube current on each tube was 25 ma. The heavy glass of the tube walls and 5 mm. of bakelite of the tube shields gave the filtering value of 0.2 mm. copper shield. The output intensity was 7210 r per minute, distance 9.5 cm. Females were placed in gelatine capsules for treatment. All breeding was carried on at about 30° C. Lethal dose [2] is the lowest dose used after which no eggs have hatched.

When dose was fractionated, it was divided into three periods of approximately equal length. Two-hour intervals were allowed between exposures for experiments on hatchability, one-hour intervals for those on androgenetic males.

Observations

In order to demonstrate the significance of new data, some results, previously published, must be summarized briefly. Studies (Whiting, 1945a) on hatchability of 6824 eggs x-rayed in metaphase I have demonstrated that, when they are unfertilized, (a) the survival cure is exponential, (b) lethal dose is about 2400 r, and (c) hatchability is not changed significantly by variation of intensity of treatment, fractionation of dose or delay in oviposition. When these eggs are fertilized after treatment by untreated sperm their hatchability is not significantly changed. It was concluded, therefore, that death is due to dominant lethal effects which arise from single irreversible events. Eggs, irradiated in this stage, and fertilized by untreated sperm, may give rise to diploid females if dose is sub-lethal or to an occasional androgenetic male at either sub-lethal or lethal doses (Whiting, 1946b).

Cytological study (Whiting, 1945a) shows that chromosome fragments (terminal deletions?) may be present in the first meiotic division after treatment in metaphase I, that these fragments increase in number with increased dose, and that chromatin bridges (resulting from sister-chromatid union?) are present in the second meiotic division. These bridges are permanent and, since the egg pronucleus remains attached to them, it is pulled into a "tear-drop" as it moves inward. In unfertilized eggs, it undergoes cleavage and, in fertilized eggs, it usually contacts the male pronucleus and fuses with it. The diploid nucleus then divides and shows clearly the difference between the two groups of chromosomes, treated and untreated. In both cases, chromatin bridges are present in cleavage divisions and death of embryos ultimately occurs. Occasionally, however, the egg pronucleus is retarded to such a degree that the sperm pronucleus divides without it and a normal, haploid androgenetic male is formed (Whiting, 1948). Androgenesis results, then, from structural changes in the x-rayed maternal chromosomes of the type which, when less extreme, cause death to gynogenetic males and to females.

[2] Apparent inconsistencies in lethal doses in successive papers dealing with Habrobracon eggs are due to changes in method of calibration at the Marine Biological Laboratory. Conditions of treatment have not varied. In this paper all doses have been corrected for the latest measurements.

Hatchability studies on 12,634 eggs irradiated in prophase I (Whiting, 1945a) have demonstrated that, when they are not fertilized, (a) the dose-action curve is exponential only at lower doses and that, after higher doses, response increases at a disproportionate rate, (b) lethal dose is about 54,000 r, and (c) hatchability is not changed significantly by variation of intensity of treatment or delay in oviposition but is increased at high doses by fractionation of dose.

Prophase I eggs, free from dominant lethal changes after exposures to sub-lethal doses of x-rays, and fertilized by untreated sperm, develop into females. No androgenetic males have been produced by them. Cytological study of divisions after treatment in this stage has shown (Whiting, 1945b) that fragments, bridges, or both may appear in either meiotic division but that bridges, rarely present in the second meiotic division, are single and do not retard the egg pronucleus. No androgenetic males develop in these eggs, therefore, because of the absence of mechanical hindrance to free movement of the egg pronucleus. Even after very heavy treatments, eggs often fail to show any chromosome aberrations.

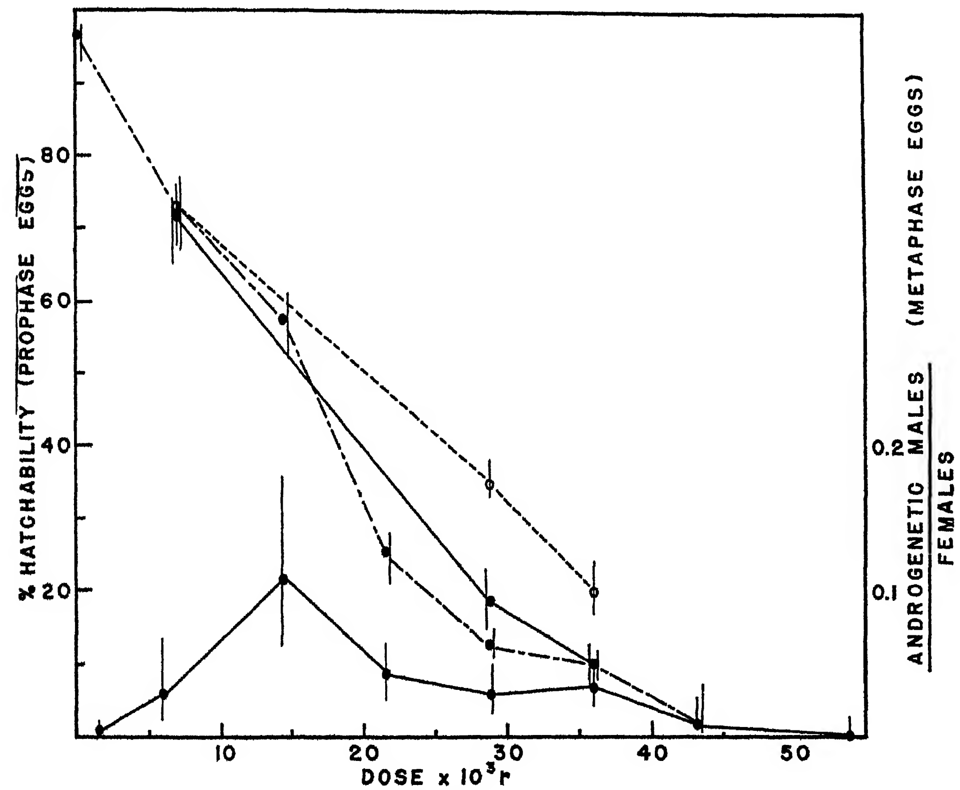

FIGURE 1. Lowest curve, $\frac{\text{androgenetic } ♂♂}{♀♀}$, plotted against dose. Remainder are dose-action curves for hatchability of unfertilized prophase I eggs. Continuous treatments (●), two experiments (—·—·—·) and (————); fractionated treatments (O), (- - - -). 95 per cent confidence interval is indicated for each experimental value.

Lowest dose after which significant reduction in hatchability was recorded was 50 r for metaphase I and 850 r for prophase I.

In Table I are assembled the data on the occurrence of androgenetic males in relation to dose. Results produced under constant experimental conditions are summarized in section A of the table and it is these which are expressed graphically in Figure 1. To be noted especially are the facts that (a) although androgenetic males develop only in eggs x-rayed in metaphase I (lethal dose for egg nucleus about 2400 r), they occur after treatment with doses up to that lethal for prophase I eggs

TABLE I

Androgenetic males in relation to dose and number of females x-rayed. A. Females from wild type stock No 33, experimental conditions carefully controlled. B. Miscellaneous females, experimental conditions not controlled.

	Dose in r units	No. ♀♀ treated	No. androgenetic ♂♂	Androgenetic ♂♂ / ♀♀ Treated
A	675–2700	1015	5	0.0049
	6000	192	6	0.0313
	14,420	146	16	0.1095
	21,630	469	20	0.0426
	28,840	487	15	0.0308
	36,050	533	19	0.0356
	43,260	358	4	0.0111
	54,075	344	1	0.0029
		3544	86	0.0243
	64,169–144,200	359	0	
	64,169	181	0	
	64,890(frac.)	97	2	0.0206
		4000	88	
B	6000	65	1	
	12,000	150	4	
	14,420	623	6	
	40,000?	52	3	
	50,000?	89	0	
	50,000?(frac.)	87	8	
	Total	5066	110	

(lethal dose about 54,000 r), and (b) that androgenetic males per treated female increase with dose up to about 15,000 r and then decrease, and (c) that fractionation of dose permits development of androgenetic males at doses lethal to them when dose is administered continuously. Data on fractionation and androgenesis are not extensive but in each of two experiments one androgenetic male was produced in comparison with none from 181 females treated with continuous dose. In two experiments listed in section B a similar difference between eggs exposed to continuous and fractionated treatments was found. Dose was not accurately measured but was probably about 50,000 r.

The chance that the array of ratios in section A, column 4, is a random variation of uniformity in expectation is infinitesimal according to the χ^2 test ($P = 0.000000$).

Facts relevant to the discussion of cytoplasmic injury are expressed graphically in Figure 1. They fall into two categories, those related to hatchability of unfertilized eggs x-rayed in prophase I (x-rayed chromosomes in x-rayed cytoplasm) and those related to incidence of androgenetic males (untreated chromosomes in x-rayed cytoplasm). The 95 per cent confidence interval for each experimental value is plotted in the figure. Tables of confidence of Ricker (1937) and of Clopper and Pearson (1934) were used.

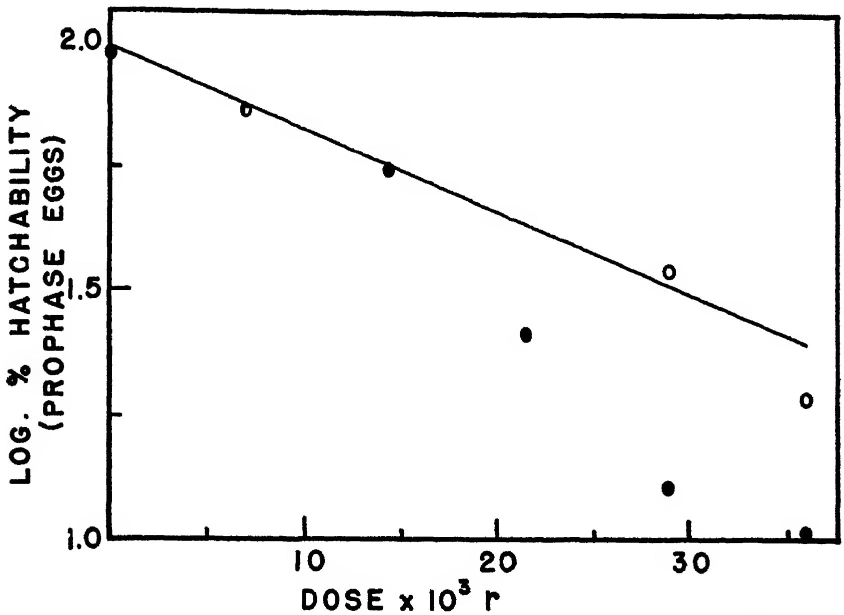

FIGURE 2. Percentages of hatchability of unfertilized prophase I eggs plotted semilogarithmically against dose; control and continuous treatments (●), fractionated treatments (○). By method of least squares a straight line was fitted to data of untreated, one minute (continuous + fractionated), two minutes (continuous), four minutes (fractionated) and five minutes (fractionated). For significance of experimental values consult Figure 1.

For hatchability of eggs x-rayed in prophase I with continuous treatment, there are two dose-action curves; with fractionated treatment, one. The former curves are not exponential, the latter is (Fig. 2). Note that the dose at which response to fractionation becomes apparent is about 15,000 r.

The curve representing androgenetic males per female rises to about 15,000 r and then falls until the lethal dose for them is reached. A steadily increasing number of chromatin bridges with resultant increase in retarded egg pronuclei is to be expected with increase of dose, so that androgenetic males should increase steadily with dose if there were not a concomitant increase of some factor which reduces their viability. Since this factor is x-ray induced, it must be cytoplasmic.

Several questions may have occurred to the reader and these are discussed at this point.

Why are there so few androgenetic males, even at doses optimal for their occurrence? Is there a high mortality of androgenetic embryos and, therefore, some permanent cytoplasmic injury at all doses? A comparison of androgenetic males per metaphase I eggs laid (6 381) with androgenetic cleavage per metaphase I eggs studied cytologically (6/291) shows that the difference between these two groups is not significant at the dose ranged used, 14,420 – 28,840 r. The small number of androgenetic males is due, then, not to the death of many but to the fact that very few egg pronuclei are sufficiently retarded to permit development of sperm nuclei alone (Whiting, 1948).

How do androgenetic males compare in vigor, viability, fertility and mutation rate, with gynogenetic males produced by the same females and with untreated controls? Detailed data on this subject are being prepared for publication, but in summary it can be stated that there is a striking difference between androgenetic males and gynogenetic males from the same females. All but one of the 110 androgenetic males found have been perfectly normal, vigorous, and fully fertile. The single exception died as a pupa. No visible mutations have appeared in them. They are indistinguishable from untreated controls in every way. Gynogenetic males tend to die as larvae or pupae and survivors have shown a significantly higher percentage of visible mutations, 29 among 417 or 6.95 per cent. Using the exact method of treating contingency tables (Yates, 1934), it is found that the chance that these percentages, 0 and 6.95, are variants of uniformity in expectation is very low ($P = 0.00188$).

Do heavily irradiated females lay fewer eggs than those treated with lighter doses? If so, this would reduce ratio of androgenetic males to mothers. Detailed analysis (Whiting, 1945a) showed that, after any dose up to 150,000 r, the average number of metaphase I eggs per female is the same as for controls.

Do sperm enter heavily irradiated eggs as freely as controls and do they move about in the egg normally? X-rayed females mate readily after any dose and, after sub-lethal doses, produce the expected ratio of daughters.

Are the cytological phenomena of the x-rayed egg nucleus the same at very high doses as at low? No accurate counts have been made of relative numbers of fragments, bridges, retarded pronuclei, etc., at very high dose but all these conditions have been observed in many eggs after treatment of metaphase I with 60,000 r.

Discussion

Evidence for chromosomal injury as the cause of death of Habrobracon eggs irradiated in metaphase I, with doses up to 2400 r, is convincing. The survival curve is exponential, and cytological study has demonstrated that ootids without chromatin fragments also give an exponential dose-action curve (Whiting, 1945a; Lea, 1946). This curve is higher than actual hatchability and suggests that some ootids without fragments are inviable. This can be explained by failure to see small fragments in some eggs. Such a failure is understandable in view of the small size of Habrobracon chromosomes and, therefore, still smaller size of the fragments and the granular nature of the yolk in which they lie. It is highly probable that each egg that fails to hatch has at least one fragment in it.

That death of x-rayed metaphase I eggs is due to chromatin loss through terminal deletions which act as dominant lethals (Muller, 1940; Pontecorvo, 1941) has been accepted by Lea (1946) and he discusses the similarity of these results to those of Sonnenblick (1940) on Drosophila eggs. Lea writes, after a detailed discussion of the subject (1947), "It is evident that dominant lethals in unfertilized eggs, as well as in the sperm, can be explained by lethal types of structural change." From the data on hatchability of x-rayed metaphase I eggs of Habrobracon, he estimated that there are 1.7 breaks primarily produced per 1000 r per haploid chromosome complement and that all breaks are permanent (Lea, 1946). This accounts for the extreme sensitivity of these cells by a method consistent with facts and with theories accepted for the explanation of similar responses of sperm cells to x-rays.

Evidence for causes of death of Habrobracon eggs irradiated in prophase I, with doses up to 54,000 r, suggests that both chromosomal and cytoplasmic changes are involved. The study of the nature of chromosomal injury is complicated by the type of dose-action curve which is exponential only for the first three points (including controls) (Fig. 2). There is no response to fractionation at these doses. Eggs, exposed to lethal doses, have been seen with chromosomal aberrations although many seem to lack them. This does not preclude their presence, however. The conditions of the chromosomes at time of treatment would allow for many types of chromosomal rearrangements, and their identification is more difficult than that of the relatively large fragments seen after irradiation in metaphase I. The increased response of x-ray prophase I eggs to higher doses suggested induction of a new phenomenon at about 15,000 r. This was interpreted (1945a) as due to an increase in multiple-hit, complex rearrangements which would increase rapidly at higher doses (Sax, 1938) and complicate the curve. Many types of multiple-hit chromosomal changes are viable, however, and would not be expected to reduce hatchability to the extent indicated in these experiments.

If the rise in mortality of both androgenetic and gynogenetic males induced by doses above 15,000 r were chromosomal in nature, it would be necessary to postulate that injury (breaks) in metaphase chromosomes decreases while that in prophase chromosomes increases above that point in response to increased dose. There remains the possibility that stickiness of chromosomes replaces breaks but this would increase androgenesis since it is permanent after high doses in the forms in which it has been reported (Sax, 1942). It is unlikely to occur in prophase chromosomes. There is no evidence for it in Habrobracon eggs irradiated with doses up to 60,000 r. If cytoplasmic injury is not involved it would also be necessary to suppose that fractionation of dose increases injury in metaphase chromosomes but decreases it in prophase chromosomes. One-hit chromosomal breaks (terminal deletions?), characteristic of metaphase injury, do not respond to fractionation of dose.

The first appearance of change in response to dose at about 15,000 r, its effect on reducing survival of gynogenetic and androgenetic males, its response to fractionation in increasing both types of males, combine to indicate that this change is due to cytoplasmic injury.

In Figure 2 are plotted semi-logarithmically percentages of hatchability of unfertilized x-rayed prophase I eggs. By the method of least squares a straight line was fitted to the data giving hatchability of untreated, one minute (continuous and frac-

tionated), two minutes (continuous), four minutes (fractionated) and five minutes (fractionated) of treatment. For the last, percentage of hatchability is below expectation on the basis of one-hit permanent changes. One fraction of treatment at this dose was longer than any others used and perhaps this reduced recovery. Curve for hatchability after continuous treatments at high doses is clearly not exponential. All data suggest that, if proper conditions of fractionation are found, a good exponential curve of hatchability can be obtained which will represent the effects of dominant one-hit irreversible chromosomal changes only since cytoplasmic injury will be prevented. Under such conditions, the number of androgenetic males should increase steadily with increase in dose and percentage of visible mutations should be the same for both continuous and fractionated treatments. Lethal dose for prophase chromosomes should be over 90,000 r.

X-ray induced chromosomal changes have been explained by some investigators as "direct-hit" changes due to the production of ionization in particular molecules and this is known as the "target theory" (Giese, 1947; Lea, 1947). Criteria of the validity of this theory are (a) independence of response to change in intensity or to fractionation, (b) absence of a threshold for response, (c) dependence of response or wave length and (d) an exponential survival curve (for one-hit aberrations). Results of irradiation of metaphase I eggs clearly support this theory. Unfortunately, no experiments have been carried out with radiations of different wave lengths.

When cytoplasmic injury is eliminated by fractionation at high doses, the resultant phenomena likewise support the target theory of chromosomal change in eggs x-rayed in prophase I.

In contrast to chromosomal change, cytoplasmic injury has a rather high and definite threshold.

Does the cytoplasmic injury in itself prevent the androgenetic embryo from maturing or does it influence the untreated chromosomes so that they are incapacitated? Untreated male pronuclei in cytoplasm x-rayed with 70,000 r look normal. The consistent absence of any injury in surviving androgenetic males favors the view that cause of death of the embryo is directly cytoplasmic.

Two facts have been noted about heavily irradiated eggs: (1) when laid they are softer and more flexible than control eggs and (2) the first cleavage nucleus, whether haploid or diploid, tends to be situated more posteriorly than in control eggs. These facts suggest decrease in cytoplasmic viscosity.

To this author, the situation which exists at four minutes of treatment, 28,840 r, represents an ideal one for testing effects of environmental changes on cytoplasmic injury and thereby, perhaps, obtaining some clue as to its nature. Any response similar to that of fractionation of dose would indicate reduction of cytoplasmic injury.

Summary

Wild type Habrobracon females were x-rayed and mated to untreated recessive males. Two kinds of haploid males were produced, gynogenetic (x-rayed chromosomes in x-rayed cytoplasm) and androgenetic (untreated chromosomes in x-rayed cytoplasm).

Eggs x-rayed in metaphase I × untreated sperm

Gynogenetic males develop from the unfertilized eggs, androgenetic males from fertilized. Death of the former and origin of the latter are caused by different degrees of the same type of x-ray induced chromosomal injury. Dose-hatchability curve for gynogenetic males is exponential and their lethal dose is that of the chromosomes in this stage, about 2400 r. Percentage of androgenetic males increases up to about 15,000 r, then gradually decreases until none is produced at about 54,000 r which is the lethal dose for the cytoplasm in this stage. Percentage of androgenetic males can be increased at doses above 15,000 r by fractionation.

Eggs x-rayed in prophase I × untreated sperm

Gynogenetic males develop from the unfertilized eggs. No androgenetic males develop due to absence of type of chromosome aberration necessary for their formation. Dose-hatchability curve for gynogenetic males is exponential up to about 15,000 r, after which it falls at an increased rate. It can be restored to an exponential curve by fractionating dose. Lethal dose is about 54,000 r. This is the lethal dose for cytoplasm. That for the chromosomes in this stage is considerably higher.

Chromosomal vs. cytoplasmic injury

Some androgenetic males survive after dose over twenty times greater than that which is lethal for chromosomes of eggs in which they develop. At all doses they resemble the controls and differ significantly from gynogenetic males in visible mutation rate, viability, and fertility. The changes peculiar to gynogenetic males must be chromosomal in origin since both types of males develop in irradiated cytoplasm. Evidence suggests that these changes are directly induced and supports the target theory of chromosomal injury.

Since there is no evidence for chromosomal injury in surviving androgenetic males, the reduction of their number at doses above 15,000 r, through embryonic death, must be directly cytoplasmic.

The increase in survival of both gynogenetic and androgenetic males in response to fractionation of dose must be due to reduction of cytoplasmic injury since they have only x-rayed cytoplasm in common.

Conclusion

At doses from 50 r to about 15,000 r, death of Habrobracon eggs (one stage more sensitive than sperm, the other less so) is due to chromosomal injury. It is not reduced by fractionation of dose or changes in intensity.

Above 15,000 r, x-rays induce cytoplasmic injury which may exert a lethal effect on developing embryos. It is reduced or prevented by fractionation of dose.

Injured cytoplasm has an "all or none" effect. It may kill embryos but does not induce visible mutations in untreated chromosomes or reduce fertility or viability of survivors. Its expression resembles, therefore, dominant lethal genetic effects and it acts directly in killing the embryo and not indirectly through injury to untreated chromosomes.

Evidence supports the target theory of chromosomal injury.

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PROGRAM AND ABSTRACTS OF SEMINAR PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1949

July 12

Plus and minus mutations in biochemical requirements in Salmonella typhimurium. H. H. Plough, Madelon R. Grimm and Martin L. Vogel, Amherst College.

For more than two years we have been studying biochemical mutations in *Salmonella typhimurium* and this work is being continued at the Marine Biological Laboratory. The work is of interest like that on *Neurospora* and on *Escherchia coli,* because it appears to be uncovering some of the basic biochemical activities of genes. In addition in *Salmonella* the serological or antigenic variants are so well known that it seems possible to attempt some correlated study of biochemical mutants and antigenic variants.

Our method is to expose living organisms in quartz flasks to ultra violet, or at Woods Hole, to X radiation in doses up to 15,000 R units. Then these are diluted and plated according to Lederberg's method on agar containing the essential salts and an energy source, glucose. After incubation the plates are layered with a complete medium, and re-incubated. Small colonies which appear after the second layer are often constant mutant strains which are found to require one or more amino acids or vitamins as essential growth factors.

By this method we have found many mutants of which the most frequent are strains requiring cysteine, proline, histidine and thiamin. One freshly isolated strain required thiamin, and this has mutated to a strain which is thiamin independent. Thus mutations occur in both plus and minus directions. Energy utilization in relation to reduced sulfur in the cysteine-requiring strain is being studied.

Evidence that response to fractionation of x-ray dose in Habrobracon eggs is cytoplasmic. Anna R. Whiting. No abstract submitted.

X-ray mutations and fecundity of Mormoniella. P. W. Whiting and Marion E. Kayhart. No abstract submitted.

July 19

Reversible enzymic reduction of retinene to vitamin A. Alfred F. Bliss.

Fresh solutions of bleached visual purple form vitamin A (Bliss, *Biol. Bull.* 1946). Morton and co-workers have shown that vitamin A is also formed when synthetic retinene (vitamin A aldehyde) is fed or injected into rats. Since vitamin A is an alcohol, and can be dehydrogenated to a typical aldehyde, it seemed possible that the enzyme involved might be the well-known reversible DPN-specific alcohol dehydrogenase. Acetone and ammonium sulfate precipitate of rabbit liver were prepared according to Lutwak-Mann. Bisulfite or cyanide was used as aldehyde trapping reagents to displace the equilibrium towards the aldehyde side. Crystalline vitamin A, dissolved with a detergent, Tween 80, was the substrate and coenzyme I, the hydrogen acceptor. The aldehyde formed was released by dilution or alkaline destruction of the addition compound (absorption maximum ca. 330 mμ), and extracted with petroleum ether. Experiments to date have shown up to 40% conversion to the aldehyde. Complete reversibility of the dehydrogenation is easily accomplished in the presence of enzyme and reduced coenzyme.

Wald has reported that retinene in retinal rods and extracts of whole retinas is irreversibly reduced by the retinene reductase of the rods in the presence of reduced coenzyme I. We have confirmed the activity of isolated intact rods. However, we have found that the reductase

activity of extracts of whole retinas is an artefact due to the large amount of enzyme from the non-visual part of the retina. Furthermore, we have found that vitamin A formation by isolated rods is freely reversible in the presence of cyanide.

We therefore need no longer assume a closed visual cycle to explain the formation of retinene from vitamin A. Instead, it is probable that the dehydrogenation is accomplished by alcohol dehydrogenase with the formation of visual purple which acts as the physiological trapping compound for retinene.

Some effects of ultra-violet light on the catalase activity and on photosynthesis of Chlorella pyrenoidosa. A. FRENKEL.

It has been demonstrated by Arnold (1933) that ultra-violet light ($\lambda = 2537$ Å) inhibits the light reaction of photosynthesis, as he could show that the percentage inhibition was the same in flashing and in continuous light.

On the assumption that ultra-violet light could also have affected the oxygen liberating catalyst in photosynthesis, the catalase activity of Chlorella cells was tested before and after irradiation with ultra-violet light. In each case an increase in the catalase activity was found (Table 1), a phenomenon which had been observed by Euler (1933) with irradiated yeast cells. No catalase was released by the cells, as the suspending fluid showed no activity after the cells had been centrifuged off.

TABLE I

Measurements were performed with 0.026 cm.^{3} (wet volume) of *Chlorella pyrenoidosa* cells suspended in 3 ml. of 0.035 M $KHCO_3$ and 0.065 M $NaHCO_3$ at 25° C.

Time of exposure to ultra-violet light. Incident intensity 1.3 ergs./mm.^{2} sec.	Photosynthesis in per cent of control	Rate constants (sec.$^{-1}$) of decomposition of H_2O_2 by 1 cm.^{3} (wet volume) of Chlorella cells
0 minutes	100	1.3
3 minutes	80	1.5
6 minutes	50	2.5
11 minutes	0	9.8

Arnold had observed that the absorption spectrum of chlorophyll does not change after irradiation of Chlorella suspensions by ultra-violet light. We have noticed, however, that in intact cells the transmission of the red chlorophyll peak increased after the ultra-violet light treated cells were exposed to visible light. This bleaching of chlorophyll, for a given dose of ultra-violet light, is a function of the intensity and of the time of exposure to visible light. In some way the energy transferring mechanism in photosynthesis has become uncoupled and the light energy directly or through some photoperoxide produces the bleaching of chlorophyll.

Biochemical properties of succinoxidase from salmonella aertrycke.[1] ERNEST KUN.

Succinoxidase was obtained by lyophilization of the washed insoluble residue of the micro-organisms, which were previously sterilized at 60° C. three times for twenty-five minutes. This enzyme catalyzed the oxidation of succinate by molecular oxygen. K_s was found to be $2.2.10^{-3}$ M succinate, heat of activation of succinate oxidation 10.470 cal. per moles of succinate. Cytochrome C inhibited the aerobic oxidation of succinate, the type of inhibition being of competitive nature. Cytochrome C was reduced by the enzyme, which reduction was not influenced by succinate. Since cytochrome oxidase was not found to be present in the preparation, it was assumed that molecular oxygen was activated by a different catalyst than the cytochrome system. Some evidence was obtained that H_2O_2 is formed during succinate oxidation by way of a flavin-like catalyst. The succinoxidase preparation had catalase, peroxidase, and fumaric hydrogenase activity; the latter not being inhibited by inhibitors which completely stop succinic dehydrogenase activity such as malonate and iodoacetamide. It seems possible that in the course of the preparation of the Salmonella succinoxidase the cytochrome oxidase is denatured and a flavoprotein which is associated with the succinoxidase system can serve as acceptor of molecular oxygen.

[1] Kun, Ernest, and Abood, L. G.: *J. Biol. Chem.* 180, vol. 2, in press.

An enzymatic product with acetylcholine-like activity, derived from brain extract. DAVID NACHMANSON, S. HESTERIN, H. VORPHAIEFF. No abstract submitted.

JULY 26

Evidence for activity of desoxyribonuclease in nuclear fusion and mitosis by the use of d-usnic acid. ALFRED MARSHAK.

A crystalline substance, d-usnic acid $C_{18}H_{16}O_7$, was isolated from the lichen *Ramalina reticulata.* It was found to inhibit the growth of several species of bacteria, but was especially active against the human tubercle bacillus. It also prevented cleavage and completely inhibited uptake of radioactive phosphorus by the fertilized eggs of Arbacia, but had no effect on their oxygen consumption. It was found that in the presence of usnic acid ($10\,\gamma$/ml., a dose sufficient to completely inhibit cleavage), the sperm penetrated the egg and the sperm nucleus reached the surface of the egg nucleus at the same time as in the controls (8 to 12 minutes after fertilization). However, the normal fusion with the egg nucleus and dispersion of the Feulgen-positive material of the sperm nucleus did not take place. This suggested inhibition of the system desoxyribonucleis acid (DNA)—desoxyribonuclease (DNase). The activity of DNase (crystalline) and DNA isolated by the method of Gulland (N/P equals 1.66) in the presence and absence of sodium usnate was then investigated using viscosity change as index of activity. It was found that $10\,\gamma$ of the usnate could completely inhibit the enzyme action, and that this inhibition required the presence of cobalt ($CoCl_2$). Cobalt in the absence of usnate gave no inhibition of the enzyme.

Unlike streptomycin, usnic acid did not form complexes with DNA so that its action was probably on the enzyme rather than the substrate.

Since usnic acid also inhibited cell division if added to fertilized eggs after the prophase of the first cleavage was initiated, it follows that mechanisms for the dispersion of DNA such as DNase are involved in the mitotic cycle as well as in the fusion of sperm and egg nucleus.

The growth and metamorphosis of the Arbacia punctulata pluteus. ETHEL BROWNE HARVEY.

The well known three- or four-day pluteus of Arbacia with two long anal arms and two short oral arms and bright red pigment spots will develop no further at Woods Hole unless specially fed. The best food is the diatom, *Nitzchia closterium,* which is cultured on Miquel's solution. The later development of the pluteus and metamorphosis is described with photographs. In about ten days after fertilization a new pair of arms with red tips grows out and later another pair of long arms. The body of the adult grows up inside the pluteus. In about two months, the five primitive ambulacral feet appear, and then primitive spines between the ambulacral feet. The animal tumbles about on its arms as well as swims with its cilia. In about two and a half months, after reaching full development, the arms begin to go to pieces, by resorption and by breaking off. The body of the adult grows larger inside the pluteus, and metamorphosis occurs in about four months after fertilization the later stages taking place very rapidly. The young adult is about 0.5 mm. in diameter.

The pluteus from the centrifuged egg develops in the same way. The pluteus from the white half-egg obtained by centrifuging is at first colorless and smaller than that from the whole egg. It acquires the red pigment spots in three or four days, and if fed develops in the same way as the whole egg and is similar in size and pigmentation.

Motion pictures showing the reactions of cells in frog tadpoles to implants of tantalum.[1] CARL C. SPEIDEL.

The reactions of cells in the tail of the frog tadpole to implants of tantalum have been recorded by cine-photomicrography. The same implants have been kept under observation for

[1] Aided by a grant from the American Cancer Society (Committee on Growth). The tantalum was supplied by the Ethicon Suture Laboratories, New Brunswick, N. J.

as long as several months. Motion pictures have been taken at both normal and low speeds. Cell movements are especially well revealed by the low speed picture.

Pictures of implants of tantalum powder show the early responses of leukocytes, endothelium of lymph and blood vessels, epithelium, and fibroblasts. Cells of these types near the implant may pick up tantalum granules. Leukocytes are most active in this respect. Frequently a cutaneous papilla forms after 2 or 3 days at the site of an implant. It grows out into a finger-like structure carrying some of the tantalum implant with it. It becomes eliminated after several more days by pinching off at the base.

Tantalum-laden macrophages often enter lymph or blood vessels and are carried away. Other tantalum-laden macrophages remain at the implant site for long periods (the motion pictures record this up to 84 days). Such cells exhibit continual slow adjustments. Encapsulation of tantalum and tantalum-laden macrophages often occurs after about 12 days, particularly in the case of deeply located implants. In later stages the capsule shell may become somewhat fibrous.

Tantalum wire implants are surrounded during the first day by a thin film of leukocytes. These isolate the wire from the adjacent tissues which display very little inflammation. Short lengths of tantalum wire thus walled off persist indefinitely. The surrounding leukocytes seem to form a fairly complete syncytial shell after a few days.

Effects of temperature upon survival of newborn guinea pigs subjected to anoxia. JAMES A. MILLER. No abstract submitted.

AUGUST 2

Labile P in nucleic acids. ABEL LAJTHA.

There is a striking parallelism between muscle and other organs, for example kidney and liver. If we let rabbit muscle or any other kind of muscle stand at room temperature, the ATP in it is gradually split; and parallel with the decreasing ATP concentration, the elasticity of muscle fibers decreases, a stiffness gradually develops (rigor mortis), and the solubility of the highly viscous muscle protein actomyosin decreases too.

The post mortem changes in kidney are analogous. If we mix fresh minced kidney with strong salt solutions, a greatly viscous extract is obtained, and the sticky solution shows a strong double refraction of flow. If, before extraction of the kidney, we let it stand at room temperature for about half an hour, the viscosity of the solution will be very small, there will be no DRF and it will not appear sticky—showing that only very small amounts of the kidney structure-protein went into solution if any at all.

In the muscle the changes are mostly restored by adding physiological amounts of ATP. Even large amounts of ATP do not restore the lost solubility of structure proteins in kidney. The analysis shows that compared with the muscle, there is only about one tenth as much ATP in the kidney.

The question arises whether in kidney the nucleic acid plays the role played by ATP in muscle. The first question in approaching this problem is whether nucleic acids contain labile P.

Nucleic acids were prepared from kidney and liver in three different ways. In a set of experiments emphasis was laid on purity of the product, in another on quantitative yields, and in the third on mildness of the method avoiding all possibility of hydrolysis.

Working with rabbits the animal was killed by decapitation, the abdomen immediately opened, the organs excised and within three minutes after the death of the animal the organs were in the Waring blender.

To prepare pure nucleic acids the organs were washed with cold trichloracetic acid, then with lipoid solvents, and with strong NaCl, reprecipitated with acids at pH 2.5 several times, and washed with lipoid solvents again several times. As in the other methods followed, the purification was made with pentose and desoxypentose tests and with ultraviolet absorption spectra. With this type of reprecipitation we get pure nucleic acids very fast, and working at a low temperature we can retain almost all labile P.

To get quantitative results I extracted the organs with hot NaCl solution containing 5 per cent Na_2CO_3. Extracting three times for about fifteen minutes the analysis of the remainder

showed that about 98 per cent of the P containing compounds were dissolved. Precipitation was made complete with the combined action of acid and alcohol. This method, however, must be corrected, as experiments with nucleic acids prepared in another way and after being boiled in basic NaCl solution showed that about 10 per cent of the labile P is split by 45 minutes boiling in a salt solution containing 5 per cent Na_2CO_3.

To work as fast as possible and retain all labile P groups, the organ was washed with cold alcohol, then with water, and then extracted in many ways. One of the methods used for example was extracting it with hot water. All the extracting solutions were then analyzed for nucleoprotein P and labile P afterwards.

The results of these experiments are that the nucleic acids of kidney and liver contain a labile P which is hydrolyzed in normal acid in 10 minutes and which amounts to about 20 per cent of the total P.

This would show that for approximately every tetranucleotide unit there is one labile P in the nucleic acid.

The accumulation of phosphate and evidence for synthesis of adenosinetriphosphate in the fertilized sea urchin egg.[1] EDWARD L. CHAMBERS AND WILLIAM E. WHITE.[2]

Unfertilized and fertilized eggs of *Strongylocentrotus purpuratus* were prepared in 0.2 per cent suspensions in sea water containing 0.060–0.100 mg. P per 1000 ml., maintained at 15° C. The concentration of inorganic phosphate in the suspension fluid was determined at intervals using the Deniges-Atkins method.

The concentration of inorganic phosphate in the sea water overlying the unfertilized eggs underwent a slight increase, while that overlying the fertilized eggs underwent a marked decrease. Similar results were obtained using the eggs of *S. franciscanus.*

Other experiments, based on the above findings, were done with P^{32} as $P^{32}O_4$ added to a 0.2 per cent suspension of fertilized eggs. The rates of disappearance of P^{32} and of P^{31} from the medium were found to be identical. Also, the rate of uptake of P^{32} by the eggs was found to correspond exactly with the loss of P^{31} from the medium. These data indicate that the rapid uptake of P^{32} by fertilized eggs (Brooks and Chambers, *Biol. Bull.*, 95, 1948) is due to an accumulation of phosphate within the egg while the slow uptake of P^{32} by the unfertilized egg represents an exchange process.

The rate of uptake of phosphate, in the concentration range of 0.015–0.100 mg. P/1000 ml. sea water, was 0.003–0.004 mg. P/ml. fertilized *S. purpuratus* eggs/minute.

The inorganic (IP) and labile phosphorus (LP) in the 5 per cent trichloracetic acid extracts of unfertilized eggs and of fertilized eggs at 70–80 minutes after insemination (50 per cent cleavage time at 15° C. being 110 min.), were determined by the method of Borbiro and Szent-Györgyi (*Biol. Bull.*, 96, 1949). The distribution of P^{32} in the fractions was also determined. Labile phosphorus is defined as the P hydrolyzed in the presence of 1 N HCl at 100° C. in 10 minutes. The non-hydrolyzed P represents that fraction which remains in the TCA extract after extraction of the inorganic and of the labile P with isobutyl alcohol.

The results are presented in the accompanying table.

In the fertilized eggs at 70–90 minutes after insemination there was observed, in the TCA extract, a marked decrease in the inorganic P fraction and an increase of labile P. The increase of labile P was greater than the decrease of inorganic P and was due undoubtedly to penetration into the egg of phosphate from outside. The labile phosphorus has been identified as P split from adenosinetriphosphate (White and Chambers, Revue de pathologie comparée et d'hygiène générale. In press).

In the unfertilized and fertilized eggs 95–96 per cent of the total P^{32} was found in the acid-soluble extracts. As seen in the table 86 per cent of the P^{32} in the acid soluble extract was found in the LP fraction of the fertilized egg, indicating that the major portion of the phosphate which entered the eggs was incorporated in adenosinetriphosphate. These results indicate that,

1 Aided by a grant from the N.C.I., U.S.P.H.S.

2 University of California, Berkeley, New York University, and the Eli Lilly Research Laboratories, M.B.L., Woods Hole, Mass.

Distribution of P and P^{32} in the acid-soluble extracts of unfertilized and fertilized eggs

	Mg. P Ml. eggs			% Total P^{32} in acid-soluble extract			Relative specific activity		
	IP	LP	IP + LP	IP	LP	Non-hydrolyzed P	IP	LP	Non-hydrolyzed P
Unfertilized	.059 mg.	.410 mg.	.469 mg.	18%	73%	8%	1.0	.49	(.08)
Fertilized	.028 mg.	.451 mg.	.479 mg.	8%	86%	6%	1.0	.65	(.07)

following fertilization, a synthesis of ATP occurs at the expense of inorganic P. The decrease in inorganic P is accompanied by the entrance of P from the external medium.

Some methods of producing traveling contraction nodes in adult frog skeletal muscle fibers (motion picture). B. A. Cookson [1] and Floyd Wiercinski.

Small bundles of muscle fibers, teased from the adductor magnus of *Rana pipiens,* were immersed in a solution containing 1.3 per cent NaCl and approximately 0.7 volume per cent H_2O_2. After approximately 2 minutes small contraction nodes were seen forming in various regions of the fibers. These nodes usually recurred in the same regions at regular intervals. After originating, they traveled along the fibers. Usually the node as it formed split into two nodes traveling in opposite directions. Frequently the nodes collided and canceled out. Sometimes contraction nodes were formed which involved only half the circumference of the muscle fiber.

Reduction of the NaCl concentration to 65 per cent (approximately isotonic with frog's blood) resulted in an absence of response. The response could be restored by increasing the osmotic pressure through the addition of non-electrolytes such as 1.5 per cent (by volume) glycerol or 7 per cent sucrose. The response was not lost when 1.6 per cent KCl was substituted for 1.3 per cent NaCl.

A slight increase in the H_2O_2 concentration produced, instead of traveling contraction nodes, large stationary areas of fiber which rhythmically contracted and relaxed. During the contraction the sarcolemna became wrinkled. A slight decrease in the H_2O_2 concentration resulted in a complete absence of response. Approximately .002 per cent 2-methyl naphthoquinone (a powerful anti-choline acetylase) could be substituted for 0.7 volumes per cent H_2O_2.

Certain concentrations of 2-methyl naphthoquinone in .65 NaCl produced asynchronous contractions of the myofibrils. Upon making the NaCl solutions hypertonic these asynchronous contractions became first more vigorous and then synchronized as the usual traveling contraction nodes involving the whole fiber.

A solution containing 1.3 per cent NaCl, 0.75 volumes per cent H_2O_2, 1 per cent glutathione and enough 0.1 N NaOH to adjust the pH to 7.4 was found to keep its potency for over 2 days, whereas the solutions containing only 1.3 per cent NaCl and 0.7 volumes per cent H_2O_2, due to the instability of the H_2O_2, remained potent for only 2 to 3 hours. With the former solution it was possible to produce traveling contraction nodes in isolated fibers with injured ends.

Investigation on muscle fibers. Andrew G. Szent-Györgyi.

The material used was the psoas muscle washed with glycerol as described by Dr. A. Szent-Györgyi (*Biological Bulletin,* 96, 140, 1949). The question was how far the behavior of these fibers corresponds to that of actomyosin threads. The actomyosin thread contracts, in the presence of ATP at low salt concentrations up to 0.2 M NaCl or KCl. At higher salt concentrations it dissolves and dissociates into actin and myosin. The contraction of the glycerinated

[1] This work was done while one of us (B. A. C.) was holding a Cancer Fellowship of the National Institute of Health, U. S. Public Health Service.

fibers is independent of salt concentration. They contract maximally even at 0.5 M NaCl, the highest salt concentration used. The close packing and the prolonged washing in glycerol stabilize the actomyosin in the fibers so strongly that ATP cannot dissociate it even at high salt concentrations.

If the structure is loosened by high concentration of ions which have specific dissociating action (sodium pyrophosphate, $NaHCO_3$, NaOCN) for a few minutes, the white, opaque and brittle muscle becomes transparent and slightly elastic. The contraction is maximal between 0.1 and 0.25, M NaCl. Below and above this salt range there is no contraction, the difference being very sharp. The fibers behave after the above treatment like the actomyosin thread.

The effect of the treatment can be reversed by immersing the muscle into 0.1 M NaCl for a few minutes.

The results indicate that the contraction of the pyrophosphate treated muscle takes place in at least two steps. The first is actomyosin formation, which depends on salt concentration. The second is the contraction itself, caused by the ATP only after actomyosin was formed.

On the structure of fibrin clots. Elemer Mihályi.

Several investigators of the late nineteenth century reported the solubility of fibrin clots in urea solutions. Wöhlisch and his co-workers, however, could not confirm this finding.

The problem has considerable importance because the protein gels and coagula, where the particles are bound by secondary forces, are all soluble in urea solutions. Insolubility may thus be an indicator of co-valent bonds between the fibrin molecules.

In the experiments which will be described, the solubility of fibrin in concentrated urea solutions was definitely confirmed. When urea is dialysed out, the clot is reconstituted. The pH of the fibrin solution during the dialysis decides whether a coarse or a fine type gel will be formed.

The viscosity of fibrin in 30 per cent urea solution is normal and equal to that of fibrinogen in similar conditions. The pH has no influence on the viscosity. At 20 per cent urea concentration the viscosity is increased by increasing the pH up to 8.6. Further increase of pH again decreases the viscosity. At still lower urea concentrations the increase of viscosity on alkalinisation leads to gelification.

The solubility of fibrin in urea solutions makes it possible to investigate in Tiselius apparatus the electrophoretic mobility of this protein. If the action of thrombin involves some of the ionising groups of fibrinogen, a study of the differences in electrophoretic mobilities between fibrinogen and fibrin may give some information on the nature of the process of clotting.

It was found that fibrin has a lower mobility above and a higher mobility below its isoelectric point than fibrinogen. The isoelectric point of the two proteins are very close together. In 20 per cent urea solution fibrinogen is isoelectric at pH 5.5, fibrin at 5.6. The results indicate that in the zone alkaline to the isoelectric point the net charge of fibrin is lower than that of fibrinogen.

August 9

Hemolytic action of anionic detergents. Lois H. Love.

The anionic detergent, sodium dodecyl sulfate, acts as both a hemolytic and anti-hemolytic agent. The course of hemolysis is complex, being very rapid for a few seconds and then stopping or slowing greatly, often for many minutes, before the remaining cells hemolyze. The cells which survive the initial, rapid hemolysis can be made to hemolyze rapidly by procedures which would be expected to remove detergent from the cells (dilution or the addition of $BaCl_2$).

The effect of temperature reflects this complexity. For each detergent concentration there is a temperature at which hemoylsis is slowest. The effect is so large that an increase of 1° C. can change the hemolysis time from less than 1 minute to more than 1 hour.

The pH effect depends on the detergent concentration. With high concentration the hemolysis time decreases from pH 6.0 to 8.0. With low concentrations the order is reversed. The results with temperature and pH appear to be due to different effects on the hemolytic and protective stages of hemolysis.

Sodium tetradecyl sulfate acts like sodium dodecyl sulfate if complications due to its low solubility are avoided. It is also shown that the presence of micelles can alter the course of hemolysis.

How simple are the so-called "simple hemolysins"? M. H. JACOBS, CAROLYN M. STOUT, MARIAN W. LEFEVRE AND W. E. LOVE.

Included among the so-called "simple hemolysins" are substances like soaps, bile salts, and saponin the behavior of which is so complex as to cause difficulty in explaining and frequently in repeating experimental results. Further evidence of this complexity is the appearance in the literature of terms such as "catastoichic," "zone phenomenon," etc. to describe behavior that has not been satisfactorily explained.

A clue to some of the complexities of behavior of the substances in question is provided by a study of a truly simple hemolysin, butyl alcohol, which we have previously shown (*Biol. Bull.*, 77, 319, 1938; 93, 223, 1947) to be capable of producing a condition of extreme and irreversible cation permeability that leads to swelling, rather than to the normal shrinkage, of erythrocytes at alkaline reactions. Like butyl alcohol, sodium oleate can also be shown to produce a condition of cation permeability. In the frequently discussed system: erythrocytes, oleate and alkali, the factor that determines whether the alkali shall be protective or destructive is not whether it is added before or after the oleate, but rather whether it is added before or after the cells have been made cation permeable by the oleate.

Complicating and sometimes obscuring the action of this factor is another to which we have recently directed attention (*Federation Proc.*, 8, 80, 1949), namely, the antihemolytic effect of agents like soaps and bile salts, which by forming a layer at the surface of the erythrocyte prevent the escape of hemoglobin under conditions where it would otherwise occur. The relation of this type of action to the "zone phenomenon" is obvious. In addition to these two factors, several others now under investigation further complicate the situation, but appear to be no less capable of accurate analysis than those here mentioned.

An analysis of the photoelectric method of measuring permeability.[1] F. R. HUNTER.[2]

In an attempt to avoid some of the difficulties of interpreting data obtained using the hemolysis or the swelling technique, an analysis was made of Wilbrandt's shrinking technique. This consists of equilibrating erythrocytes in a solution of non-electrolyte in Ringer Locke. An aliquot of cells containing the non-electrolyte is then transferred to a salt solution and the rate of shrinking as the non-electrolyte leaves the cells is measured photoelectrically. Series of shrinking curves can be obtained by using non-electrolyte solutions of various concentrations and by measuring shrinking in salt solutions of different concentrations. As would be expected, a greater volume change is obtained by increasing the concentration of non-electrolyte or decreasing the concentration of the salt solution in which the cells shrink. Hematocrit determinations showed that chicken erythrocytes behave as perfect osmometers (using a b value of 0.355 which is the dry weight) when placed in salt solutions of tonicities between R.L. and $2 \times$ R.L. The galvanometer deflections of the apparatus are linearly related to volume changes over this same range of salt concentrations. There is some deviation of observed from calculated volume changes of cells equilibrated in the non-electrolyte-R.L. solution and allowed to shrink in a salt solution. Spectrophotometric data show that this deviation is due in part to slight, initial hemolysis. However, since this hemolysis occurs initially, rather than terminally as it does when swelling measurements are made, the shrinking data are more easily interpreted and constancy of time for one half the galvanometer was obtained from day to day. It is believed that if chicken erythrocytes are equilibrated in 0.6 M non-electrolyte in R.L. and allowed to shrink in 1.875–1.625 $\times$ R.L. solutions, a change in time for one half the total deflection is an indication of a permeability change.

[1] This work was supported by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service and the Faculty Research Fund, the University of Oklahoma.

[2] Department of Zoological Sciences, the University of Oklahoma, Norman.

Potassium and sodium exchange in rabbit erythrocytes treated with butyl alcohol. A. K. PARPART AND J. W. GREEN. No abstract submitted.

AUGUST 16

Studies on degenerating testicular cells in immature mammals. I. Analysis of degeneration in primordial male germ cells and in a hitherto undescribed germ cell in albino rats aged one to nine days. EZRA ALLEN.

In the early postnatal albino rat testis three waves of degenerating cells have been reported. The first involves the so-called "primordial" germ cells. These are found in the central part of the testis cords. They are quite abundant in the 1-day testis, gradually decrease in number till very few if any remain at 9 days of age. The second and third waves of degeneration take place with the development of the spermatocytes and the spermatids. (Hoven, 1914; Firket, 1920; Hargitt, 1926.) The details of the degenerative process of the primordials are described in this paper as well as the degeneration of another type of cell called a "large" cell.

I. Degeneration of the primordial cells. Shortly before birth these cells become vacuolated. At birth their cytoplasm is still vacuolated. Vacuolation proceeds until none remains, and the cell wall has disappeared. Meanwhile the chromatin breaks up into small particles which persist as the nucleus decreases in size. These are described as pycnotic cells. At the age of 7 days the pycnotic cells have practically replaced the primordial complete cells. Gradually the pycnotic cells lose their chromatin and finally are absorbed. A few remain at the 9th day of age.

II. The "large" cells. The large cells differentiate from the basal cells of the sex cords, beginning to appear at 3 days. At metaphase they are about the size of the full-sized primordial cells. Their chromosomes are large and well defined. Cell division may occur normally, or at meta- or anaphase the chromosomes may break up into irregular large and small bodies which lie in a dense mass of achromatic substance. These cells become two to three times the diameter of their normal state at metaphase, and are spoken of as "giant" cells. Their chromatin bodies become progressively smaller and finally are lost to view in a delicate matrix which resembles the matrix of the central part of the sex cord; absorption follows. Most of them have been absorbed at 9 days. The large cells are not very numerous. A few have been found with close to the haploid number of chromosomes. One showed clearly either 21 or 23 chromosomes. Generally in the giant cells the number was close to or at the diploid.

Comparative study of lipids in fish. CHARLES G. WILBER.

A review of the pertinent literature indicates that little information is available concerning the amounts and the types of lipids present in fish, especially Arctic aquatic forms. Specimens of *Pygosteus pungitius* were collected in Arctic Alaska during the summer of 1949. They were analyzed for total fatty acids, cholesterol, and phospholipid. Similar analyses were made on 2 groups of guppy (*Girardinus guppyi*). The mean values for the Arctic fish in per cent fresh tissue are: fatty acid, 4.88; cholesterol, 0.32; phospholipid, 1.87. For the guppies these values are: 8.87, 0.56, and 2.31 respectively. It is obvious that there is more cholesterol and fatty acids in the guppies than in *Pygosteus*. Statistically, the phospholipids in either fish are the same. Ratios of the various lipids in the different fish were calculated with the following results: there is more phospholipid in relation to cholesterol or to fatty acids in the Arctic fish than in the guppy. This fact may be interpreted as indicating a higher level of fat turnover in the former than in the latter. The ratio of cholesterol to lipid phosphorus is lower in the stickleback than in the guppy. This low ratio indicates a decreased amount of water in the tissues of the Arctic fish, a fact which may be correlated with the resistance of the Arctic fish to freezing injuries.

The structure of insulin and the cyclol hypothesis. DOROTHY WRINCH.

The x-ray diffraction study of wet crystalline insulin (Crowfoot and Riley, *Nature* **141**: 521, 1938) yields many intensities. If the phases were known, which they are not, the atomic pattern of the crystal could be directly calculated. However, from the Fourier transform of

the observed intensities, a precisely equivalent "experimental" vector map can be calculated. There is therefore a second way to use such data as clues to protein structure, if we can interpret the language of vector space. Accordingly x-ray data enable us to assess the validity of the cyclol or any other hypothesis of protein structure, if, but only if, we can discover the characteristic features of the "synthetic" intensity or vector maps (Wrinch, *J. Chem. Phys.* **16**: 1007, 1948) expected from the given hypothesis.

Last year it was reported that all cyclol cages have intensity maxima at ca. 10–11½A (*Biol. Bull.* **95**: 272, 1948). This established a contact between the cyclol hypothesis and the intensity data for many proteins. In the present note a further contact is claimed between the hypothesis and the published section of the experimental vector map of wet crystalline insulin (Crowfoot, *Russian Chem. Jour.* G, 15, 215, 1946).

We may visualize wet crystalline insulin as a three-dimensional network of molecules, arrange in particles, in a water medium. It is inconvenient and unnecessary to construct the corresponding vector map because of the many water entries. Instead we reduce the entire crystal to the water level, replacing the water by zero entries and the protein molecules by *reduced* molecules. The resulting vector map is then the true vector map reduced to the corresponding level. To obtain it, we enter the vector maps of each reduced molecule on itself at the origin, the vector maps of each on every other at appropriate points. There results a map which for a certain domain about the origin represents simply the superpositions of self-interactions of each reduced molecule, the rest of the map being predominantly a highly complex manifestation of the molecular pattern of the particles, from which no information regarding the structure of the molecules can be obtained.

Given a C_1 cage skeleton, or twin skeletons on cube or tetrahedral faces, of density d, with emergent R-groups of density d_r and interior of density d_i, reduced to the water level d_0, it will be shown elsewhere that the gross structure is manifested in characteristic manners in vector space. When d is larger than d_0 and d_r, d_i are sufficiently smaller than d_0, the central trigonal section has a quasi-hexagonal *hill* region about the origin, enclosing and enclosed by *valley* regions. If a is the average of C—C and C—N bond lengths in the cyclol skeleton, say 1.5 A, the edge of the outer hexagonal boundary of the hill region is ca. $4a\sqrt{6} \sim 14.7$ A.

The corresponding section of the experimental vector map of wet crystalline insulin (Crowfoot, loc. cit.) also has about the origin, a *hill* region, approximating a hexagon, with outer edges within an angstrom or less of 14.7 A. Furthermore this region likewise encloses and is enclosed by *valley* regions. The direct relationship between the two maps is confined to this valley-hill-valley region about the origin. However, it is this region alone which gives information as to the gross structure of the molecules in the insulin particle.

It is therefore claimed that contact is now established between certain typographical features of the published section of the experimental vector map and certain topographical features of the corresponding section of the synthetic vector map derived from a molecular skeleton or twin skeletons having the size and shape of the C_1 type of structure proposed for the skeletons of the molecules of insulin.

(This work is supported by the Office of Naval Research under contract N8onr-579.)

The development of Menidia-Fundulus hybrids. J. M. MOULTON. No abstract submitted.

GENERAL MEETINGS

Development of spermatozoa in albino rat from 9 to 50 days of age. EZRA ALLEN.

The age of 9 days marks the stage when the primordial germ cells have practically all been absorbed. There remain only the indifferent basal cells which give rise to several generations of cells by meiosis, terminating with the mature sperm. Some degeneration accompanies the process.

I. Differentiation of the sperm. The first step in the transformation of the basal cells is a well-marked change in their appearance. At 12 days the chromatin gathers into rather large unequal masses, in preparation for mitosis, which occurs at 13 days. The daughter cells become leptotene cells. By 14 days, leptotene cells form; at 15 days they become abundant. Pairing

and splitting of chromosomes follow. By 18 days or a little earlier the pachytene stage is reached. No further differentiation takes place for about 8 days. The epithelium increases in number of layers and the pachytene cells increase proportionately. At 26 or 27 days metaphase is reached; at 39 days sperm is present in some tubules; at 42 days in nearly all tubules, but none in the lumens. At 48 days they have matured and are passing into the epididymis.

II. Degeneration phenomena. Degeneration is accomplished in a number of ways. 1. A tubule at any age between 14 days and maturity may lose all of its basal cells except the outermost layer, which usually does not degenerate. 2. Up to 44 days, exfoliation into the lumen of normal or degenerate cells. 3. At 14 days, tubules which lie next to the rete may discharge their cells into the rete. 4. Several types of degenerate cells may form in the epithelium. (a) Cells with a few rather large pycnotic bodies. (b) Cells with a large number of tiny pycnotic bodies. (c) Cells with a large number of dense granules surrounded by dense cytoplasm. (d) These pass into a small cell with structureless matrix. (e) Vacuolation of different types of germ cells. (f) Multinucleate giant cells.

On the source of birefringence within the striated muscle fiber. WILLIAM R. AMBERSON, R. DALE SMITH, BETTY CHINN, SYLVIA HIMMELFARB, AND JOHN METCALF.

Matoltsy and Gerendas (*Hungarica Acta Physiologica* **1**: 116, 1948) claim that in rat muscle the I bands within the sarcomere contain a negatively birefringent "N"-protein which compensates for the positive birefringence of actomyosin filaments, rendering the I-bands isotropic. Similarly, Dempsey, Wislocki and Singer (*Anat. Rec.* **96**: 221, 1946) believe that negatively birefringent phospholipins in the I-bands render them isotropic. These authors used conventional paraffin technic. Barer (*Biol. Rev.* **23**: 159, 1948) discusses the validity of such theories.

It has been shown by us (*Fed. Proc.* **7**: 2, 1948) that myogen and myosin may be extracted from whole rabbit muscles using pyrophosphate to dissolve myosin without removal of actin. We have now applied K pyrophosphate solutions (5 to 10 per cent) to whole muscles moving the telson of Limulus. The relaxed sarcomeres are 8 to 10 micra long. After removal of divalent cations, pyrophosphate solutions are applied, at 0° C. and pH = 10.5. The A-band birefringence disappears in from one to four days. Histological detail is well preserved with Z-lines and myofibrils still visible. In the extracts a viscous protein appears, exhibiting flow birefringence. Presumably a protein, responsible for the normal birefringence, has been removed from its loci in the A-band, in or on the myofibrils.

In these extractions we have never observed the development of negatively birefringent areas, so long as the tissues are handled in aqueous solutions. In paraffin sections we have occasionally seen zones of negative birefringence, often in the I-band, but we consider this phenomenon to be an artefact.

Tiselius analysis shows complicated electrophoretic patterns in pyrophosphate extracts. These patterns differ greatly from those obtained from muscles extracted with phosphate buffers at pH = 8.1, in which birefringence persists. It now appears possible to derive two different protein mixtures from these muscles, only one of which contains the protein responsible for birefringence.

Echinochrome: its absorption spectra; pK_1' value; and concentration in the eggs, amoebocytes and test of Arbacia Punctulata. ERIC G. BALL AND OCTAVIA COOPER.

The absorption spectra of crystalline echinochrome ($C_{12}H_{10}O_7$) obtained from arbacia eggs as described by Ball (*J. Biol. Chem.* **114**, VI, 1936) has been determined at pH values from 1.1 to 8.5. From the data obtained the pK_1' value of the pigment at 26° C. has been calculated to be 6.38. The acid form shows three peaks located at λ255, 335, and 475 mμ with molar extinction coefficients of 1.93×10^4, 0.87×10^4, and 0.65×10^4 respectively. The ionized form also displays three peaks, but centered at λ275, 400, and 475 mμ with ϵ values of 2.11×10^4, 0.88×10^4, and 1.12×10^4 respectively. Instability of the pigment first becomes evident spectroscopically at pH 6.8 and increases rapidly as the pH is raised. Crystalline pigment obtained from the tests has the same properties as the egg pigment.

Fresh aqueous extracts of eggs or the amoebocytes of the body fluid of either males or females yield absorption spectra similar to those of crystalline echinochrome. Though the pigment in such extracts is largely bound to protein, it shows the same shift in its absorption spectrum with pH changes as observed for the crystalline pigment. This would seem to indicate that the ionizable hydroxyl group of the pigment is not involved in linkage to the protein carrier.

Acid alcohol extracts of eggs, amoebocytes, spines, or 2 to 2½ day old plutei (obtained through the courtesy of Dr. E. B. Harvey) yield the same absorption spectrum, indicating that the pigment from these different sources is identical. Eggs contain on the average 0.58 g. of pigment per 100 cc. of eggs packed by centrifuging. The amoebocytes, whether from males or females contain 3.78 g. per 100 cc. of packed volume of body cells. The test contains 0.19 g. of echinochrome per 100 g. The average female arbacia can thus be calculated to contain a total of 38 mg. of echinochrome; the male has about half this amount.

Effect of ultraviolet radiation on the rate of cell-division of Arbacia eggs. H. F. Blum, J. P. Price, J. C. Robinson, and G. M. Loos.

Appropriate dosage with ultraviolet radiation (wavelengths ~2700Å to 3130Å) slows the rate of cell-division in *Arbacia* eggs, without permanent damage. Recovery of normal rate is gradual and complete. Under comparable conditions the rate of recovery is about the same if the ultraviolet radiation is applied before fertilization, between fertilization and first cleavage, or between first and second cleavages. Application of ultraviolet within about 20 minutes prior to a given cleavage fails to delay that cleavage, but does delay subsequent ones. This tends to obscure the smooth character of the recovery process, which goes on whether cell-division takes place or not.

Recovery is markedly accelerated by illumination with "visible" light (principally blue-violet, ~4000Å to 5000Å), after dosage with ultraviolet. The white halves of centrifuged eggs exhibit the same recovery phenomenon. Visible light does not enhance recovery if applied before dosage with ultraviolet, nor does it accelerate the rate of cell-division of normal eggs.

Except that visible light does not enhance the recovery, similar results are obtained when x-rays are applied to the eggs in appropriate dosage. This important exception indicates, however, a fundamental difference in the mechanism of action of ultraviolet radiation from that of x-rays.

The tolerance of stenohaline forms to diluted sea water. Marie Boyle and Maxwell S. Doty.

T. A. Stephenson[1] has pointed out that at Nanaimo, British Columbia, there is an abundance of normally open ocean forms in waters of estuarine concentrations. To test the hypothesis that coldness of the water resulted in a tolerance of the organisms to such dilutions several species of algae were exposed, in lots of 15, to 0.8 times the normal concentration of sea water and to the normal concentration (about 33%) at various temperatures ranging from 6° C. to 31° C. When the experiments were over the total injury at elevated temperatures was greater in the case of thalli exposed to dilute sea water than in the case of those exposed to normal sea water. At low temperatures there was either no injury or the injury was considered to be the same for both the thalli in diluted and normal sea water. Thus since organisms such as *Sphacrotrichia divaricata* (C. Ag.) Kylin, sporelings of *Fucus vesiculosus* L., and others can tolerate dilution better in colder water an explanation may be provided for the occurrence of stenohaline forms in some brackish waters.

Reversal of polarity in limbs of urodele larvae. Elmer G. Butler.

The polarity of a urodele limb can be reversed by a method of transplantation so that the original proximal end of the limb becomes the free end of the transplant. Regeneration of such a reversed limb will take place. The operative procedure of reversal consists in the insertion

[1] 1948. Report on work done in North America during 1947–1948. Privately printed in Grt. Brit. by Neill & Co., Ltd., Edinburgh.

of the distal tip of a limb, after amputation of the hand, into a pocket on the body wall just posterior to the shoulder. After initial healing into the pocket has taken place, the limb is then amputated slightly below the head of the humerus, thus permitting the original proximal region of the upper arm to swing free as the distal end of the transplant. Larvae of *Amblystoma opacum* and *Amblystoma punctatum,* 20 mm. to 45 mm. in length have been used. Fifty-two regenerates have been obtained and studied; forty-one other cases have been fixed for study of histological changes preceding regeneration; fifteen transplants underwent extensive regression.

Following the amputation through the upper arm, regression of the transplant begins distally and continues usually until the elbow joint is reached. Then, either a blastema is established and regeneration proceeds, or the transplant assumes the character of a non-regenerating appendage. In the latter case amputation of the tip of the transplant, in particular the removal of a remnant of the humerus at the original elbow level, results in the initiation of regenerative activity.

Within the regenerate, terminal portions of radius and ulna are formed carpals, metacarpals and phalanges are established resulting, in many cases in the development of a well formed hand with four digits. These experiments demonstrate clearly, therefore, that the original proximal end of a forearm, when it becomes the free end of a reversed limb, possesses the capacity for the establishment of a blastema in which are organized the normal components of the distal structures of a limb.

Rhythmic alterations in certain properties of the fertilized Arbacia egg. ROBERT CHAMBERS, EDWARD L. CHAMBERS, AND LAWRENCE M. LEONARD.[1]

The experiments were done on eggs which had been denuded of their extraneous coats, viz., the fertilization membrane and the so-called hyaline plasma layer. The removal of these coats was brought about by placing the eggs, 1½ minutes after insemination, into 0.95 M urea for 1 minute and then transferring the eggs to a mixture of NaCl (18 parts) and KCl (2 parts) isotonic with sea water. In this mixture the eggs develop and undergo cleavage at about the same time as in sea water.

As is well known, the cytoplasm of the fertilized egg undergoes successive changes of its structural and physical framework which cannot be considered simply in terms of changes in overall viscosity. The changes are: (1) the formation within 6 to 8 minutes of a gelated cortex in which the pigment vacuoles collect as they migrate peripherad from all parts of the interior; (2) the development of the growing aster at 10 to 15 minutes accompanied by an extending gelation, which finally involves all of the cytoplasm except for the central "lake," the radial canals and the periphery of the aster beneath the cortex; (3) an interphase during which the cytoplasm is fluid and contains the diminutive nuclear spindle; and (4) shortly before cleavage, the growth of the amphiaster which constitutes a spreading gelation around the two polar regions of the spindle.

The experimental procedures consisted of (1) compressing the eggs in a hanging drop between the overlying slide and underneath a strip of coverslip fastened on the upturned shaft of a coarse microneedle, (2) rupturing the eggs either by tearing with microneedles or by applying a drop of high surface tension inert oil which induced a rupture to permit outflow of the interior, and (3) inducing swelling by immersing the eggs in a 50 per cent solution of the NaCl/KCl mixture.

The following results were obtained: 1. Compression of the eggs, irrespective of the stage of development, resulted in a flattening to 2 or 3 times the original diameter without causing rupture. The flattening process induced disappearance of the asters and caused breaks in the gelated cortex which solated without a rupture of the protoplasmic surface film. It is significant that when the eggs were beginning to cleave a protoplasmic film separated away from the contracting gelated band of cortical material at the furrow. The constricting band persisted for a time after the egg had been converted into a very much flattened disc.

2. Sudden rupture of the egg induced disappearance of the asters but in this case a difference was noted between the astral phases and the interphase. When ruptured during the astral phase the resulting sol state was such that the granular contents of the eggs flowed out rapidly. The

[1] Under grant of the NCI, USPHS; New York, University and the Eli Lilly Research Laboratories, M.B.L., Woods Hole, Mass.

cortex of the egg remained gelated and shrank into a diminutive ball of condensed pigment vacuoles. When ruptured during the interphase there was little or no outflow. Evidently, during this period, the cytoplasm maintains a viscosity sufficiently high to prevent an outflow whereupon the exposed cytoplasmic vacuoles undergo agglutination.

3. Exposing the eggs to hypotonic solutions give indications that the swelling is not accompanied by the disappearance of the asters. During the period of the gelated monaster and amphiaster phases the eggs swelled relatively slowly (determined by serially timed photographs) and 80 to 100 per cent of the eggs burst within 5 minutes. Of significance is the fact that during the late amphiaster when the cleavage furrow was forming the bursting occurred at the furrow where the cortex is at the maximum of stiffness. On the other hand, during the early fluid phase (within 4 to 5 minutes after insemination) the swollen eggs did not burst. More detailed studies were made on the later fluid phase, i.e., the interphase between the monaster and amphiaster stages. During this interphase the swelling occurred relatively rapidly and to larger proportions, but the eggs did not burst even after prolonged exposure.

Evidently the physical state of the egg interior and particularly that of the cortex conditions the bursting in the hypotonic solution.

Theobromine and theophylline effects upon rate and form of Arbacia development. Ralph Holt Cheney.

Dimethylated dioxypurines differing only in the position of a CH_3 group were used. Gametes were shed into dishes with different concentrations of the drug-in-sea-water for 15 minutes, then mixed for fertilization. Developmental rate and form in SW and TbSW or TpSW were compared with controls. Experimental molarities included one concentration of approximately maximum solubility. Observations were made at intervals during a three-day period.

All eggs per experiment were shed by one female and all sperm from a single male. Four combinations of untreated and treated gametes were utilized in mixing for fertilization. The combinations were as follows: N♀ × N♂, N♀ × Tb♂ or Tp♂, Tb♀ or Tp♀ × N♂, Tb♀ or Tp♀ × Tb♂ or Tp♂, all mixed and developed in SW. The last two combinations were also mixed and developed in TbSW or TpSW.

Results demonstrate that the immersion of the gametes for 15 minutes prior to mixing did not render the eggs non-fertilizable and subsequently did not destroy the ability of the sperm to fertilize. Evidence suggests that pretreatment of both gametes in theobromine did not have any significant effect since development in SW was equivalent to controls. Gametes pretreated with theophylline, however, were affected, at least by higher concentrations; since, although developed in SW, the fertilized eggs showed definite retardation and failed to develop plutei.

Comparison of the effects of equivalent molarities indicate that Tb is more effective than Tp in retarding the rate of Arbacia development. Both retarded the rate primarily during the gastrula-prism-pluteus sequence. Effects are directly proportional to the concentration of the drug. Comparison with the trimethylated dioxypurine, caffeine, as shown by the author in 1948 (*Biol. Bull.* **94**: No. 1, 16–24), indicates the order of the decreasing effectiveness of these compounds upon the developmental rate and form of *Arbacia punctulata* is theobromine, caffeine, and theophylline.

Inhibition of cleavage in Arbacia eggs and of phosphorylation in cell-free egg extracts by nitro- and halo-phenols. G. H. A. Clowes, A. K. Keltch, C. F. Strittmatter and C. P. Walters.

The effects of a number of substituted phenols on oxidative phosphorylation by the cell-free particulate enzyme system of Arbacia described in the previous abstract have been measured. Typical results are shown in the table; α-ketoglutarate was used as substrate. Oxygen consumption (c.mm.) and phosphorus esterified (micrograms) are per flask (equivalent to 0.1 ml. eggs) per hour. Negative values for phosphorus esterification denote a net increase in inorganic phosphorus.

The effects previously reported for the living eggs (Clowes and Krahl, *J. Gen. Physiol.* **20**: 145, 173, 1936) parallel those upon the cell-free system in the following respects: The phenols which stimulate respiration and block cleavage also increase oxygen use and block phosphorylation by the cell-free system; dinitrothymol blocks cleavage and phosphorylation but reduces oxygen consumption in both systems; o-nitrophenol and picric acid are inactive in both systems at concentrations up to 0.001 M; the total concentration of each active agent required in the cell-free system (pH 6.9) is smaller than that required to block cleavage in sea water (pH 7.9) and the concentration of the undissociated acid form of each nitrophenol required to block cleavage is virtually identical with that required to block phosphorylation.

These results suggest that the Arbacia egg derives energy for cleavage from oxidative phosphorylation and that substituted phenols block cleavage by interfering with generation of high-energy phosphate bonds.

The authors thank Dr. M. E. Krahl for advice.

Conc. reagent	2,4-dinitro-phenol		4,6-dinitro-cresol		4,6-dinitro-carvacrol		2,4-dinitro-thymol		2,4,5-trichloro-phenol	
	O_2 use	P ester.	O_2 use	P ester.	O_2 use	P ester.	O_2 use	P ester.	O_2 use	P ester.
Moles per 1. $\times 10^6$										
None	36	126	34	131	28	104	38	122	31	102
0.13	36	117	34	119	28	93	34	67	—	—
0.25	—	—	34	112	30	87	24	1	32	107
0.5	39	117	38	68	32	67	25	−22	34	104
1	43	108	42	54	32	32	21	−30	36	104
2	42	77	40	−9	30	−19	21	−34	34	99
4	39	39	34	−22	26	−21	20	−30	36	77
8	—	—	36	−8	30	−17	—	—	37	9
16	38	−4	31	−4	26	−20	—	—	32	−18
32	—	—	—	—	—	—	—	—	30	−17

Toxicity responses of dividing nuclei of Allium as demonstrated with the Sudan black B technique. ISADORE COHEN.

The responses of Allium roots correlated with their mitotic behavior has been suggested by Levan (*Proc. Eighth Int. Cong. Genetics*, 1948) as the basis of a phytoassay in the preliminary screening of active substances. Treatment of onion seedling and bulb root tips for 4 hours and longer in 5 per cent ethyl alcohol approximately 0.005 M mercuric nitrate, 0.1 M and 0.01 M sodium fluoride followed by examination with the Sudan black B technique confirmed essentially results previously reported. The use of the Sudan black B as an adjunct in such assay is considered desirable because of the speed in obtaining the remarkably clear staining of onion chromosomes in which critical prophase stages and other important details are preserved. Comparable results are not usually obtained with a paraffin technique. The large number of nuclei freed from cells throughout the root tip permits an over-all impression of general reaction to the test substance.

Stickiness in various stages of mitosis was produced by the various substances tested. Supercontracted chromosomes produced by 5 per cent ethyl alcohol formed telophase nuclei in which the appearance of the nucleoli were retarded. Mercuric nitrate markedly altered the structure of the interkinetic nuclei producing gross reticula and, in some instances, prevented the new coiling of prophase as shown by heavily nucleinated prophase chromosomes with relic coiling. In the milder responses the chromocenters were accentuated when the interkinetic chromatin did not stain as intensely as the controls.

The use of Sudan black B in the study of heterochromatin in certain plant nuclei. ISADORE COHEN.

Following acetic-alcohol fixation a 0.5 per cent solution of Sudan black B in a mixture of formic, propionic, and lactic acids incorporating 50 per cent water stains, brown chromosomes and interkinetic chromatin in temporary smear preparations (Cohen, I., *Stain Tech.* 24: 177–184, 1949). When observed with a suitable green filter the chromosomes and interkinetic nuclei appear black while the nucleoli do not stain.

Different species of Allium are characterized by the presence of heterochromatic masses, the chromocenters, in their interkinetic nuclei (Levan, A., *Hereditas* 32: 449–468, 1946). Nuclei of the red onion are relatively free from chromocenters while the nuclei of the bunching onion (Ferry-Morse Seed Company's Evergreen Bunching) show varying numbers of chromocenters. Rapidly dividing nuclei which are heavily nucleinated in the interkinetic stages appear to be free from chromocenters. Nuclei with slower rates of division and nuclei from differentiated cells contain a varying number of chromocenters ranging from 9 to 18. Nuclei containing 14 to 16 chromocenters were more frequently encountered.

The chromocenters stain more intensely than the interkinetic chromatin. In older nuclei the chromocenters reveal an alveolar structure. Based upon the study of prophase and late telophase or early interkinesis, the chromocenters appear to represent in part the uncoiled portions of chromosomes, terminal and centromeric in case of the bunching onion. In the giant nuclei of differential stelar cells, some chromocenters become greatly enlarged and quadripartite at their free ends. Nuclei of onion seedlings killed in 5 per cent ethyl alcohol also show chromocenters with accentuated structure. As a rule, the chromocenters can not be distinguished in prophase after the relic coiling has been abolished and the contraction and new relational coiling have been initiated.

Smears made from the tips of secondary roots of sweet corn (Zea mays) showed the consistent appearance of 3 to 4 large chromocenters and varying numbers of small ones. A pair of larger chromocenters, closely associated with the nucleolus, may well be the nucleolar organizers reported by Morgan (*Jour. Heredity* 34: 195–198, 1943). The interkinetic nuclei are well-preserved and their chromonematic structure is not obscured as seen in preparations obtained with the paraffin technique.

Root tips of the lima bean (*Phaseolus lunatus*) were similarly examined. The interkinetic nuclei contain on the average 16 chromocenters which are precisely stained with Sudan black B. The chromocenters are connected to very fine chromonemata which are poorly stained, if at all. In prophase, the chromocenters, now visibly double, are incorporated into the long, paired chromatids. Even though the interkinetic and prophase nuclei are somewhat distorted when extruded from the obscuring basophilic cytoplasm, their isolation and the inhibition of nucleolar staining permits a critical examination of details difficult to see by other techniques.

The effect of lithium chloride and calcium low sea water on the development of the otolith of Molgula manhattensis. ARTHUR L. COLWIN.

The tadpole of Molgula normally develops a single sensory organ, an otolith, in the cerebral vesicle. This otolith appears as a black spherical structure.

If eggs in the 4- to 32-cell stages are placed in a solution of lithium chloride 1:200 in sea water for 4½ to 5½ hours, the development of the otolith appears to be inhibited and consequently absent in a very large majority of cases. Associated with this treatment is a poor development of the tadpole tail. If treatment with LiCl is not too prolonged a percentage of these tadpoles will metamorphose with the formation of branchial and atrial siphons but the otolith which is normally found between these two siphons is absent.

If eggs are placed in calcium-low sea water within 5 to 10 minutes following fertilization and remain in this solution for from 40 to 90 minutes, the tadpoles which develop may show an increase in the number of pigment spots (otoliths) ranging up to six. Each of these may be of approximately the same size, or smaller or larger than the normal one, or they may be a combination of sizes both smaller and larger than normal. These pigment spots usually appear in a group but not necessarily in the normal location; they may be found in almost any location, even lying superficially in the ectoderm and not in a cerebral vesicle. A small percentage of those treated will metamorphose but the location of the pigment spots may be other than the normal one between the two siphons.

Developmental potencies of the early blastomeres of the egg of Saccoglossus (Dolichoglossus) kowalevskyi. ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

Fertilized eggs within their membranes were treated with calcium-low sea water for varying periods before and during early cleavage. In most cases the early blastomeres separated readily and some remained apart even after being returned to normal sea water.

When the first two blastomeres were separated completely the cleavage pattern of at least the first few cleavages was similar to what it would have been as part of the whole egg. Subsequently two blastulae were formed, giving rise to two gastrulae, and eventually to two complete and perfect larvae: the transverse ciliated band, proboscis, collar, skewing of posterior end, pigmentation, and first pair of gill slits having been formed successively. The time of appearance of these structures corresponds closely with their development in the whole egg. Often complete separation of blastomeres did not occur, or the blastulae derived from separated blastomeres would rejoin. Such cases resulted in various degrees of reduplication, ranging from Siamese twins to two-headed or two-tailed monsters.

When the first four blastomeres were separated completely, the first few cleavages of each quarter appeared as they would have been as part of the whole egg. Subsequently, each formed a blastula and then a gastrula. In the best cases, each proceeded to develop into essentially a miniature of the normal-sized larva. More often the blastomeres or blastulae rejoined, producing one "twin" and two "quadruplets," or one larva of three-fourths size and one "quadruplet," or various two, three or four headed monsters with some parts enlarged or duplicated and other parts missing or reduced in size.

The above evidence suggests that at least each of the first four blastomeres is potentially capable of developing into a normal larva of proportionately smaller size.

The fertilization reaction in the egg of Saccoglossus (Dolichoglossus) kowalevskyi. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

The mature unfertilized egg of *Saccoglossus kowalevskyi* is about 420 micra long by 330 micra wide, covered by a transparent membrane about 7 micra thick. The densely opaque egg has a narrow border of fine translucent greenish granules or alveoli tightly packed and probably lying within the plasma membrane. The nucleus is in metaphase of the first maturation division.

Upon fertilization the outer membrane moves away from the egg, changing little in appearance. An inner, second membrane becomes apparent and also rises, successively thickening, wrinkling, thinning and smoothing out to a tough spherical structure.

Simultaneously with membrane elevation a wave of agitation sweeps the greenish granules, immediately followed by a wave of "boiling" during which knob-like protuberances arise over the entire periphery of the egg. Each knob consists of an opaque basal mound covered by the greenish granules, now shiny and sending projections outward as if giving off some substance. As "boiling" subsides a fertilization cone forms, similar to, but larger than, one of the knobs. Its clear outer portion presses the second membrane tightly against the first and a thread-like structure appears, extending through both membranes, and connecting cone and sperm head. The thread seems to draw the sperm into the cone. Then the opaque base recedes but a transparent "exudation cone" persists briefly and sometimes even enlarges before it withdraws. The whole process is completed within about 10 minutes of insemination. Meanwhile the greenish granules of the unfertilized egg appear to have given rise to a third extraneous layer consisting of large clear granules and entirely unlike the smooth clear membranes 1 and 2. This third layer is at first separated from the egg by a narrow transparent area crossed by minute striations; later during gastrulation it moves out to lie close to membrane 2, where it persists for some time after the larva starts to rotate.

Cytological investigations of the gut epithelium in haploids and diploids of Habrobracon. D. S. GROSCH AND A. M. CLARK.

Studies of internal tissues indicate that the single-cell layer comprising the wall of the abdominal enlargement of the digestive system should be especially suitable for morphological

and cytochemical comparisons between haploids and diploids of Habrobracon. A survey with gut tissues from a two-allele cross 25c × 25 + is being completed. The present report is based on whole mounts fixed with Gilson's fixative, stained by the Feulgen technique (Rafalko modification) and pressed flat when mounted in balsam. An observation important in the light of quantitative nucleic acid studies is that with identical technique the nuclei of "haploid" gut walls are but lightly stained after time intervals of hydrolysis and staining which leave nuclei in "diploids" well colored. This apparently indicates less DNA in the "haploid" nuclei than in the "diploid" nuclei.

Planimeter tracings of camera lucida drawings of 100 cells in each group gave mean nuclear and cell area measurements which show: (1) haploid male nuclei ($25.73 \pm 0.95\,\mu^2$) are smaller than diploid male ($39.92 \pm 1.57\mu^2$) and diploid female ($38.10 \pm 1.38\,\mu^2$) nuclei; (2) haploid male cells ($115.35 \pm 2.76\,\mu^2$) are smaller than diploid male ($173.70 \pm 7.97\,\mu^2$) and diploid female ($155.24 \pm 4.31\,\mu^2$) cells. Comparison of the nuclear per cell ratios among the three groups show them to be statistically similar (0.22 to 0.24).

Previous determinations of cell size based on studies of ommatidia size and on microchaetal counts of wing areas show larger cell areas for diploid males than for females and haploid males, with the latter two groups approximating each other. However, the cell size relationships in the present study indicate that too much reliance should not be placed on any particular tissue.

The time phase of the tide factor hypothesis. MAXWELL S. DOTY AND JUSTINE GARNIC.

The earlier published tide factor hypothesis of vertical distribution of intertidal organisms may, for testing purposes, be separated into two components: (1) elevational variations of the critical tide factors, and (2) sudden variations in the time or duration of maximum single emergence or submergence between one level and another. The latter has been investigated by exposing seventeen species of algae to each of three different conditions for periods of time varying from 2 to 96 hours. Often when the time of exposure was doubled or tripled over that time necessary to injure a few thalli, all thalli were killed. One, therefore, is led to accept the hypothesis that it is the tide factors that are responsible for the sharp upward and downward limits of intertidal organisms.

Porphyridium cruentum Nägeli and Porphyridium marinum Kylin. MAXWELL S. DOTY AND JUSTINE GARNIC.

During a study of *P. cruentum*, the terrestrial form, some of the material was accidentally dumped into a dish of sea water which upon concentrating by evaporation eventually yielded a rich culture. Following this lead it was found that when grown in 2.5 times the ordinary concentration of sea water with added nitrate and phosphate this reputedly fresh water or terrestrial form grew rather rapidly and with the characteristics attributed by Kylin to his species, including absence of the strongly unilateral sheath. Thus it seems that Kylin's species [1] (a *nomen nudum* per article 45 of the International Rules of Bot. Nomenclature) is not distinct from *P. cruentum* Nägeli.

Pioneer colonization on intertidal transects. ELIZABETH M. FAHEY AND MAXWELL S. DOTY.

Data from repopulation studies made at Woods Hole, Massachusetts, during the past few years, indicate that irrespective of the time of clearing or exposure a certain set of rapidly-growing organisms, called pioneer organisms, are the first macroscopic forms to appear. Transects cleared in the summer or fall have become populated with pioneer forms of Enteromorpha, Polysiphonia and occasionally other genera. These forms, while relatively short-lived or transient reproduce actively throughout these seasons. They are almost constantly present in the neighborhood but on the transects they may reach maturity and disappear in two months time. Late winter clearings, on the other hand, have been repopulated first by long-lived forms,

[1] Kungl., *Fysiografiska Sällskapets Lund Förhandlingar* 7 (10): 1–5, 1937.

e.g., Balanus, the reproductory periods of which are restricted largely to this part of the year. On these transects such forms as Enteromorpha failed to appear.

Slowly growing organisms, e.g., Scytosiphon, Chordaria, Nemalion, etc., tend to be the next occupants of cleared transects. They may succeed the transient pioneers on the rocks or in the case of the presence of the long-lived forms they may be epiphytic or epizotic forms, e.g., Ralfsia occurring on Balanus or the various gastropods feeding on the algae. Slowly maturing, long-lived organisms such as Fucus in time come to dominate. Since such communities tend to perpetuate themselves they may be considered as analogous to the "climax" forms of terrestrial ecologists.

The current observations tend to support the hypothesis that the course of repopulation insofar as it concerns any particular succession of species is dependent on the life cycles and forms of the organisms present as well as the time of clearing in respect to the time of reproduction particularly of the rapidly-growing longer-lived organisms. Thus, a classification of the species involved should include a consideration of at least three characteristics of the species; namely: (1) growth rate; (2) time of reproduction; (3) life cycle and life forms.

The aforementioned classification would probably be best treated in terms of a succession involving: (1) Pioneer colonization by rapidly-growing forms which may be transient as Enteromorpha or persistent as Balanus; (2) colonization by more slowly-growing, seasonal, usually non-climax forms; and (3) climax colonization by long-lived or slowly-growing forms which reproduce or reestablish themselves, e.g., Fucus and Balanus.

Intervarietal mating reactions in Paramecium caudatum. LAUREN C. GILMAN.

In collections of *Paramecium caudatum* from various localities chiefly in the states of Maryland, Pennsylvania, Massachusetts, South Dakota, and Connecticut ten varieties numbered 1 to 10 and consisting of mating types I to XVII and XX were found. So far no type XIX to go with type XX has been discovered. Each variety consisted of two mating types which gave, when mixed under the appropriate conditions, close to 100 per cent immediate agglutinative mating reaction followed by a proportionate amount of conjugation. In general conjugation occurred only within a variety although a number of exceptions were found.

In making the tests between the varieties controls consisting of unmixed samples were set up as were also controls consisting of mixtures of the two mating types in each variety. In order for the results of the mixtures to be significant it was necessary that no conjugation occur in the unmixed controls and that the control mixtures give close to 100 per cent mating reaction followed by conjugation in proportionate amounts. The amount of conjugation was estimated roughly by visual inspection of the mixtures.

No cross reactions were found with varieties 1, 4, 5, and 7. Variety 3 gave a cross reaction with variety 6. Cross reactions also occurred between varieties 2, 8, 9, and 10. The cross reactions encountered were of two kinds. The first kind consisted of a weak mating reaction involving about 5 per cent of the animals and resulting in no conjugation. The second kind consisted of mating reactions as strong as those found within a variety which were followed by proportionate amounts of conjugation. The first kind of reactions was given by the following: type VI variety 3 with type XI variety 6; type III variety 2 with type XVI variety 8; type XVIII variety 9 and type XX variety 10; type XVII variety 9 with type IV variety 2 and type XVI variety 8.

Intervarietal mating reactions in *Paramecium caudatum* reaction was given by the following: type XV variety 8 with type IV variety 2 and type XX variety 10; type XVII variety 9 with type XX variety 10. If the animals were not in optimal condition giving close to 100 per cent mating reaction at the time of mixture the first kind of reaction was completely eliminated and the second kind proportionately reduced.

The effect of temperature on growth and sexual changes in Crepidula plana. HARLEY N. GOULD.

Studies made at Woods Hole during the summer and fall of 1948 showed changes in the rate of growth and sex transformations in *Crepidula plana*. Growth of young, development of the male phase, regression of the male phase following removal from the vicinity of females,

subsequent growth and onset of the female phase are all accelerated up to the end of August. The rate of these changes falls off rather suddenly in September and progressively thereafter, communities of the limpets reaching an almost static condition by the first of December. Many factors of the environment are changing, one of which is the temperature of the sea water. The temperature of the surface water in the harbor reached 22 degrees C. in August, 1948, and decreased to 10 degrees by the first of December.

To determine the effect of temperature as distinguished from other environmental factors, parallel sets of cultures, of similar size and sexual condition were kept during July, 1949, in running sea water at different temperatures: control at 22 to 23 degrees and experimental at 12 to 16 degrees.

Small sexless young in absence of females show rapid growth at normal temperature, very retarded growth at reduced temperature. Spontaneous male development is partial at normal temperature, completely absent at reduced temperature.

Similar small sexless young confined with mature females show retarded growth at normal temperature, almost none at reduced temperature. Induced male development under these conditions is complete in most specimens at normal temperature, incomplete or absent at reduced temperature.

Males removed from the vicinity of females lose their male character quickly and grow rapidly at normal temperature, but retain male organs longer and grow slowly at reduced temperature.

It is concluded that temperature is a major factor influencing growth and sexual changes even when the summer food supply in the sea water is available.

Oxygen utilization in relation to growth and differentiation in the slime mold Dictyostelium discoideum. JAMES H. GREGG.

The growth and morphogenetic stages of the slime mold *Dictyostelium discoideum* are separate during the life cycle. The rates of oxygen consumption of the independent amoebae of the growth stage and the migrating, preculmination, and culmination stages of morphogenesis were determined. The oxygen consumption measurements were made with a microrespirometer. The independent amoebae and the individual slime molds of the morphogentic stages were analyzed for total nitrogen by a microKjeldahl method in order that the oxygen consumption measurements could be expressed in terms of a unit mass of nitrogen.

It was found that the morphogenetic stages respired at a greater rate than the growth stages. The individual slime molds of the morphogenetic stages vary greatly in size. Bonner and Eldredge (*Growth*, Vol. IX, No. 4, 1945) found that the larger slime molds culminated at a greater rate than the smaller slime molds. The oxygen consumption of the various sized slime molds was identical, within the experimental error, per unit mass of nitrogen. Therefore, the smaller slime molds require more oxygen per mm. of culmination per unit mass of nitrogen than the larger slime molds. The rate of respiration during the transition of the slime mold from the preculmination stage to the completely culminated stage was determined. The rate of respiration remained constant until the latter part of culmination when a slight decline in respiration began which continued until the slime mold ceased completely to respire. An examination of the culmination rate has shown that the culmination declines simultaneously as the respiration decreases.

It is suggested that the morphogenetic processes of the slime molds require energy above that required for growth and maintenance. In view of the parallel between the rates of culmination and respiration it is suggested that respiration and morphogenesis are coupled in this organism. This is further indicated by the fact that when morphogenesis ceases respiration also ceases.

Vitamin K as a protoplasmic coagulant and parthenogenetic agent. ATIDA HALABAN.

If protoplasmic clotting is similar to blood clotting and if protoplasmic clotting or gelation is a preliminary to the appearance of the mitotic spindle and cell division, then it might be reasoned that substances with vitamin K activity which play a role in blood coagulation might have an effect on the protoplasm of the cell and the initiation of mitosis.

The experiments were done on *Arbacia punctulata* eggs. Dilute solutions of 2-methyl-1,4-naphthoquinone were found to cause a high percentage of artificial parthenogenesis and they also produce a gelation of the egg protoplasm. Arbacia eggs were exposed to solutions of the following concentrations: 10 mg. per liter, 5 mg. per liter and 3 mg. per liter of 2-methyl-1,4-naphthoquinone in sea water for 5 to 60 minutes; they were then washed and removed to sea water at 5 minute intervals. Counts of dividing cells were made after 4 hours. An exposure of 15 minutes to the 10 mg. per liter solution gave 70 per cent parthenogenesis. Exposure to this concentration for 30 minutes results in sharp protoplasmic gelation. A 30 minute exposure to a concentration of 5 mg. per liter causes 90 per cent of the eggs to cleave. Lower concentrations require longer exposure times for a maximum percentage of cleavage.

When eggs were exposed to a concentration of 10 mg. per liter, an increase in viscosity occurred following 15 to 25 minutes of exposure and gelation was complete after 30 minutes. If after 20 minutes in the solution the eggs were transferred to sea water, there was a progressive increase in protoplasmic viscosity, so that by the end of 2 hours the protoplasm was completely gelled. Cell division followed this gelation.

Effect of ultraviolet light (2537 Å) *on cleavage time in centrifuged Arbacia eggs.* CLIFFORD V. HARDING [1] AND LYELL J. THOMAS, JR.

Arbacia eggs were centrifuged for one minute in an Emerson hand centrifuge (9000 times gravity). This treatment is sufficient to cause distinct stratification of the visible components. A small amount of isotonic sucrose was put in the centrifuge tubes to prevent crushing of the eggs against the bottom. These eggs were then placed upon a layer of isotonic sucrose (4 mm. in depth) in each of two quartz petri dishes. After two minutes the majority of the eggs became oriented with the fat cap at the top and the pigment layer at the bottom. One petri dish was then placed above the ultraviolet lamp and the other below the lamp (Westinghouse Sterilamp, 2537 Å; dose, 720 ergs per mm.^{2}). The two dishes were irradiated simultaneously. The eggs were then washed three times in sea water and fertilized about 15 minutes after the exposure. All the eggs were kept under the same conditions of temperature (approximately 23° C.) and light until counted for first cleavages. In every experiment the eggs irradiated through the fat cap (those eggs placed below the lamp) cleaved after those irradiated through the pigment layer. In experiments carried out under the conditions referred to above this difference in cleavage time averaged 11.5 minutes.

The distances of the petri dishes from the source of ultraviolet light were so adjusted that uncentrifuged eggs showed no significant differences in cleavage time between those irradiated from above and those irradiated from below. It seems, therefore, that the centrifuged eggs are truly more sensitive to the ultraviolet light when irradiated through the fat cap.

Initiation of cell division by injury substances.[2] DRUSILLA HARDING.

Extracts of injured tissues from the frog and clam were tested for their effectiveness in causing artificial parthenogenesis in the Arbacia egg. Injury substances were prepared according to the method described by Heilbrunn et al. in their studies of heat death (*Physiol. Zool.* 19: 404–429, 1946). The freezing point lowering of the solution was adjusted to slightly less than that of sea water to prevent activation by hypertonicity. The pH of the solution was between 4 and 5. In every case, the extracts caused parthenogenesis. Neutralization of the extracts, however, caused loss of parthenogenetic activity. The effectiveness of these injury substances was compared with the effectiveness of various acids at the same pH, and the following results obtained: The averages of the highest per cent cleavage from each experiment were for the extract, 27.08, for the butyric acid, 21.18, for acetic acid, 22.95, for lactic acid, 3.05, and for phosphoric acid, 1.7. The per cent cleavage in the extracts is significantly higher than in the other acids. The percent cleavage in butyric acid is not significantly different from that in acetic acid.

Viscosity determinations showed that after an exposure of the eggs to the extract just long enough to produce the first signs of division, an increase in viscosity can be detected. The vis-

[1] Public Health Service Research Fellow of the National Institutes of Health, Department of Zoology, University of Pennsylvania.

[2] Aided by a grant from the U. S. Public Health Service administered by L. V. Heilbrunn.

cosity increases about 15 minutes after removal of the eggs to sea water, and rises as much as 4-fold by 50 minutes.

It is concluded from these results that an acid substance is released as the result of injury. This acid of injury can cause an increase in viscosity and initiate cell division in the Arbacia egg.

Cell division in relation to protoplasmic clotting.[1] L. V. HEILBRUNN AND W. L. WILSON.

When a cell is incited to divide, the primary change is believed to be a protoplasmic clotting or gelation, and this clotting or gelation is thought to be essentially similar to the clotting which occurs in blood. Earlier work on the egg of the worm Chaetopterus has shown that heparin prevents mitotic gelation and also prevents division of the cell. Similar results have now been obtained with preparations of the bacterial polysaccharide which Shear found so effective in causing regression of tumors in mice and rats. When egg cells are placed in solutions of the polysaccharide 2 minutes after fertilization, mitosis is inhibited and the protoplasm instead of undergoing normal mitotic gelation remains fluid.

Other agents which influence blood clotting have a marked effect on the division of the Chaetopterus egg cell. Thus, a vitamin K, 2-methyl-1,4-naphthoquinone, in very high dilution can suppress cell division. Concentrations of 1 mg. per liter are sufficient to produce this effect. In such concentrations, the protoplasm remains fluid for hours but eventually becomes very much more viscous than normal. In higher concentrations of vitamin K, the mitotic gelation is suppressed for a time, but then the protoplasmic viscosity rises to very high levels. The eggs do not recover after such treatment.

Dicumarol also has a marked effect on cell division. A solution containing 100 mg. per liter completely stops cell division. In such a solution, the mitotic gelation is maintained and does not reverse at the normal time. The protoplasm of the cell remains more viscous for an hour or two and then the protoplasm becomes fluid and remains fluid. Typically, after a long delay the eggs in dicumarol solution undergo a single mitotic division and then stop. The effect is reversible and eggs removed from the dicumarol solution proceed to divide and develop.

Oxidative phosphorylation by a cell-free particulate enzyme system from unfertilized Arbacia eggs. A. K. KELTCH, C. F. STRITTMATTER, C. P. WALTERS AND G. H. A. CLOWES.

Preparation of the particulate enzyme system was carried out as follows: Unfertilized Arbacia eggs were washed once with 100 volumes sea water, three times with 100 volumes of a solution 0.5 M in NaCl, 0.02 M in $NaHCO_3$, pH 7.9, and collected by centrifuging 30 seconds at 1000 g. in chilled cups; all subsequent operations prior to incubation were made at 5° C. The packed eggs were mixed with 5 volumes of a solution 0.4 M in KCl, 0.01 M in sodium citrate, 0.05 M in glycylglycine, pH 7.4; this suspension was forced three times through a No. 18 needle against a glass surface, 95 per cent or more of the eggs being broken up. The resulting suspension of egg fragments was spun 8 minutes at 1000 g. in a refrigerated centrifuge (angle head). The top, pinkish-white layer was used as the particulate enzyme system effecting oxidative phosphorylation.

The concentrations of reagents in each Warburg flask were (in mM per liter): KCl, 57; citrate, 1.4; $MgCl_2$, 14; NaF, 36; inorganic phosphate, 1.4; glycylglycine, 27; glucose, 36; substrate, 10; nicotinamide, 3.6; diphosphopyridine nucleotide, 0.2; adenosine triphosphate, 0.7; cytochrome C, 0.014. The total volume was 2.8 ml., including 0.2 ml. yeast hexokinase solution and 0.6 ml. egg particulate enzyme, which was added last.

The flasks were incubated, with shaking, for 1 hour at 20° C. One ml. aliquots were fixed with 2.6 ml. cold, 11 per cent trichloroacetic acid; the inorganic phosphate was determined according to Fiske and SubbaRow. Phosphorus utilization was ascertained by comparison with appropriate controls fixed before incubation. The average initial inorganic phosphorus content of each flask was 175 micrograms, of which 96 were added. The source of the rest is undefined.

This crude particulate enzyme system can carry out oxidative phosphorylation with various substances of the tricarboxylic acid cycle as substrate. In a typical example, oxygen consump-

[1] Aided by a grant from the U. S. Public Health Service.

tion (c.mm.) and phosphate uptake (micrograms) per flask per hour were: no substrate: O_2, 24, P uptake, 13; α-ketoglutarate: O_2, 34, P uptake, 101; oxalacetate: O_2, 30, P uptake, 91; succinate: O_2, 31, P uptake, 53.

These data indicate that the Arbacia egg can generate high-energy phosphate bonds by use of substrates of the tricarboxylic acid cycle.

The authors thank Dr. M. E. Krahl for advice.

Electrographic observations on seagulls. BRUNO KISCH.

Eight seagulls have been investigated and standard leads, chest leads, and in five cases direct leads from the surface of the heart have been taken.

The heart rate of the narcotized animals (ether) is between 450 and 520 beats per minute as long as the heart beat is normal.

In each of the investigated cases the voltage of lead I is very small and lead II and III very similar, both showing a deep Q-S or a very small R and a deep S, resulting in a very definite left axis deviation. The right chest lead equals very much the left chest lead. The entire picture is very similar to the conditions previously described for the calf.

By taking direct leads from the heart surface using for an indifferent electrode, a Wilson central terminal, the cardiogram was found as follows: the right auricle shows a small R, deep S type; the left ventricle a high R, no S type. From the surface of the right and left ventricle a Q-S, or small R, deep S can be registered from the pectoral surface, but from the dorsal surface a deep Q-S.

Using the blotting paper method, auricular fibrillation and auricular flutter could be produced by application of acetylcholine to the right auricle.

The haematologic findings in the seagulls were as follows: Red blood count average 2.7 ml., maximum 3.1, minimum 2.2, haematocrit reading 34 per cent, haemoglobin content 10.4 g. per cent, size of red blood cells average $14.1 \times 8.1\ \mu$, maximum 18.3×8.6, minimum 10.8×8.1.

A detailed report with tracings will be published in "Experimental Medicine and Surgery."

An unknown type of haemolysis. BRUNO KISCH.

Among thirteen eels haemotologically investigated were three sick or dying brought in by fishermen. Only in the blood of these three animals were found, besides the normal red blood cells, red blood cells containing one or more crystal-like inclusions in the form of long prismatic or needle-like bodies. These inclusions show microscopically the same color and similar properties as the content of the normal red blood cell. In growing they stretch the membrane of the red blood cell, which reached up to twice the diameter of a normal cell (for instance: $28\ \mu$ instead of $14\ \mu$). The membrane finally ruptures. It remains a long prismatic shaped body with the attached nucleus, which retains its normal form and size. Never were these bodies found without nuclei, so probably, after the rupture of the membrane they may be dissolved, and the isolated nucleus remains.

The here described crystal-like shaped formations are probably not haemoglobin crystals because their color is not darker than the contents of the erythrocytes.

An extensive report will be published.

Genetics of Chlamydomonas—paving the way. RALPH A. LEWIN.

Problems of photosynthesis may be attacked by the methods of biochemical genetics using a chlorophyllose micro-organism which can be grown rapidly in pure culture, and in which sexual reproduction can be controlled.

Complementary mating types of *Chlamydomonas Moewusii* Gerloff have been isolated by L. Provasoli, and the life cycle can now be experimentally reproduced. The alga is routinely grown on 1 per cent agar to which mineral salts (Beijerinck's) and 0.1 per cent sodium acetate are added. Suspensions of sexually active cells can be obtained by flooding 2 to 5 day old cultures with water. The processes of clumping and pairing require light, and a minimum of 14 hours (at 470 f.c.) has been found necessary for complete fusion of gamete protoplasts. Only a very small percentage of zygotes which have been matured under continuous illumination can be made to germinate, even after a dormancy period of 1 to 2 months: many chemical and

physical treatments have been tried to "break" dormancy, without success. On the other hand, zygotes placed in darkness 14 to 28 hours after mating germinate regularly within 6 to 10 days. Zoospores are liberated on flooding, and may give rise to isolated clonal colonies. Tetrad analysis is carried out as described by Moewus (*Zeit. Ind. Abst. Verh.* **78**: 418–522, 1940).

Mutations have been obtained by ultra-violet irradiation using standard screening techniques. In addition to numerous slow-growing and palmelloid clones, the following mutants have been isolated: M.67 "Flagella-less." Sexually sterile. M.151 "Non-photosynthetic." Pigments apparently normal, but growth negligible in absence of organic carbon source. M.236 "Paralysed." Most cells unable to swim, though some may swim abnormally with slow flagellar beat. Typically flagella remain extended and rigid except for twitching of tip. Cells may progress over glass slide (2 mμ per second), drawn by peculiar serpentine movement of flagellar tip in contact with substrate. No artificial stimulation of flagellar movement achieved to date.

Genetic studies using these mutants are now in progress.

Recovery from ultra-violet light induced delay in cleavage of Arbacia eggs by irradiation with visible light. ALFRED MARSHAK.

Irradiation of Arbacia sperm with ultra-violet light 2537 Å, 52 microwatts/cm. for 30 seconds approximately doubled time for cleavage of eggs fertilized with this sperm. Increasing the ultra-violet dose increased the delay. Six times this dose given to eggs produced only 20 per cent delay, which suggests the effect is on the nucleus. Following ultra-violet irradiation with intense visible light (28,000 ft. candles) of either sperm or unfertilized eggs had no effect, neither did visible light alone affect the gametes. Visible light given to the zygote markedly reduced the delay in cleavage time. The effect was observed when the light was given 2 to 50 minutes after fertilization, but maximum efficiency was at 9 to 11 minutes. Significant reactivation was obtained with light 560 mμ–620 mμ (max. 570 mμ) (1.6 per cent total transmission), but light 330 mμ–470 mμ (max. 440 mμ) (total transmission 0.1 per cent) was more effective. Increasing exposure time gave increasing effect but a maximum was reached at 3 minutes. Following ultra-violet treatment of sperm, the delay-producing factors increased with time after irradiation. In the unfertilized egg, there was neither growth nor decay of the delaying factors. Photoreactivation was obtained with fertilized nucleated half-eggs containing no pigment granules as well as with whole eggs.

Because of their probable connection with nuclear metabolism, eggs, sperm, and zygotes were treated with the following substances before, during, and after irradiation with ultra-violet and visible light: adenosine, streptomycin, sodium usnate, folic acid, 4-amino n-methyl folic acid, and 2,4-diamino 5-p-chlorophenoxypyrimidine. None had any effect on either ultra-violet inactivation or photoreactivation. Riboflavin also had no effect on reactivation of the zygote. Sperm irradiated with visible light soon became immobile. Eggs could be fertilized before immobilization and although the sperm aster formed in the usual time, cleavage was much delayed and abnormal, but this effect seems unrelated to the photoreactivation phenomenon.

No delay in polar body formation or in cleavage was found in Chaetopterus eggs fertilized with sperm irradiated with 6 times the ultra-violet dose given Arbacia.

The effect of necrosin on the cleavage for fertilized sea urchin ova. VALY MENKIN AND LOUISE A. PIROVANE.[1]

The writer has demonstrated that injured cells of higher animals liberate in inflammatory exudates a toxic substance which per se offers a reasonable explanation for the pattern of injury in inflammation. This substance is either a euglobulin, or else it is associated with the euglobulin of particularly acid exudates. It has been termed necrosin (*Arch. Path.* **36**: 269, 1943; **39**: 28, 1945). Subsequently it was shown that this toxic substance, or one closely resembling it, is also liberated in the severely injured cells of invertebrates such as *M. arenaria* or *L. polyphemus* (*Physiol. Zoology*, **32**: 124, 1949).

These studies have been continued to determine whether necrosin has any effect on cell division of the unfertilized and fertilized ova of *Arbacia punctulata.* Necrosin has often a reduced activity when in the lyophilized state. This, however, is not always the case. The

[1] Aided by a grant from the National Advisory Cancer Council.

studies were carried on with an active dry frozen preparation, previously assayed in the cutaneous tissue of a rabbit. About 10 milligrams of necrosin were added to a standard dish containing 10 cc. of sea water and 2 cc. of suspension of Arbacia ova. The ova tended soon to agglutinate, cytolyze, or fuse. They often assumed a deep red color. In other standard dishes, 5 drops of Arbacia sperm were added, and the time and percentage of segmental division observed. It was noted in a large number of experiments that the division of the fertilized eggs was definitely retarded upon the addition of necrosin. However, once division had been initiated, the addition of necrosin failed to retard the process. The retarded division was at times, but not always, accompanied by abnormal cleavage. Necrosin was also quite toxic to the spermatozoa, so that the percentage of fertilized ova was diminished. The necrosin was originally obtained from dogs. The substitution of another canine globulin, namely the leukocytosis-promoting factor (LPF), failed to inhibit the rate of cleavage.

Mating reactions between living and lyophilized paramecia of opposite mating type.[1] CHARLES B. METZ AND EDNA M. FUSCO.

Since dead paramecia can give specific mating reactions with living animals of opposite mating type (Metz, 1946), it seemed likely that paramecia could be dried without complete destruction of mating reactivity. This proved to be the case with both *P. aurelia* and *P. calkinsi.* Reactive *P. aurelia* were killed with formalin, washed in saline, quickly frozen in a solid CO_2-acetone bath and finally dried from the frozen state (lyophilized). When suspended in saline the dried *P. aurelia* clumped strongly and specifically with living animals of opposite type. Only weak reactions were obtained with *P. aurelia* that were frozen in the living condition. *P. calkinsi* must be frozen while alive and then lyophilized. Formalin killed lyophilized *P. calkinsi* give only weak reactions. Properly dried *P. calkinsi* (but not *P. aurelia*) retain their mating reactivity for several months if not indefinitely at room temperature.

Since lyophilized *P. aurelia* not only give mating reactions but also induce macronuclear breakdown and pseudo selfing pair formation in living animals of opposite type, the activation initiating mechanism (Metz and Foley, in press) is not destroyed by lyophilization. This is in agreement with the view that interaction of mating type substances initiates activation.

Technically the procedure makes possible: (1) study of the effect of anhydrous solvents on the mating substances, (2) electron microscope study of paramecia that are known to have reactive surfaces and (3) storage of reactive animals. So far only the first of these possibilities has been explored. Thus the mating reactivity of dried paramecia (aurelia or calkinsi) is not destroyed by extraction (30 minutes, room temperature) with absolute ether, acetone, benzene or chloroform. Absolute alcohol inactivates the paramecia. These results would seem to eliminate loosely-bound fat-soluble substances as essential constituents of the mating type substance(s).

The protoplasmic viscosity of muscle and nerve.[2] PETER RIESER.

Small oil drops were microinjected into frog muscle fibers, and the rise of the drops through the protoplasm was observed in a horizontal microscope. The protoplasmic viscosity was determined from Stokes' law. An average value of 29 centipoises was obtained. Some higher values were interpreted as representing cases where a slight degree of injury had occurred. Oil drops were able to move only in one direction through the fibers. No movement across the fibers was ever observed. These facts, as well as the ovoid shape of the drops, suggest the existence of some longitudinal organization within the fibers. Microinjection of large masses of oil or air always showed that a peripheral region directly within the sarcolemma could never be displaced by these substances. By microinjection of aqueous solutions it was possible to push the protoplasm ahead through the fibers. In every instance where such a flow occurred there was a peripheral region of approximately the same thickness as obtained by the oil and air displacement method, and this peripheral region did not flow. This outer region had a minimal thickness of approximately 10 μ. The outer region thus appears to be similar to the gel-like cortex of other types of cells. Preliminary studies on oil drops injected into fibers from

[1] Aided by a grant from the National Institute of Health, U. S. Public Health Service.
[2] Aided by a grant from the U. S. Public Health Service administered by L. V. Heilbrunn.

the ventral chain of the lobster reveal the fluid nature of the axoplasm. The average viscosity was 5.5 centipoises. The shape of these drops, unlike those injected into squid axoplasm, was spherical. The oil drops were not able to move in the opposite direction, and no movement across the fibers was ever noted. In the giant axon of the squid no movement of oil drops could be observed; this is perhaps due to the greater sensitivity of squid axoplasm.

The use of extra-polar stimulus escape to measure nerve membrane characteristics. OTTO H. SCHMITT.

It is possible to show on simple theoretical grounds that a sinusoidal stimulus applied to a nerve between two ringlet electrodes should produce a signal in the extrapolar regions which decays exactly exponentially and which changes in phase linearly with distance. It is further possible to show that these two attenuation constants contain all the data needed beside longitudinal nerve resistance to determine quantitatively at each test frequency the membrane conductance, membrane susceptance, and phase velocity of propagation along the nerve. These predictions are extremely well borne out by experiment and besides verification of certain previous measurements, considerable new information regarding the effects of drugs and ions is emerging. For squid nerve a membrane capacitance of 1 to 1.5 mfd/cm.^{2} is uniformly found at 500 to 1000 c.p.s. Conductance for normal nerve in the 200 c.p.s. region is about 1 millimho/cm.^{2} but in the mid-frequency region around 500 c.p.s. total conductance of the membrane drops to near zero and seems to dip into the negative region under the influence of calcium. A strong reactive component in the 75 to 300 c.p.s. region is found which is quite sensitive to drugs and which can be pushed far into the positive susceptance region, especially by calcium and veratrine. It is also possible to demonstrate progressive changes in the membrane and response to drugs long after the nerve has ceased to respond and after conductivity of the membrane has increased many fold.

Because the method is not limited to single fiber preparations and does not require giant axons, it is foreseen as a valuable means for determining electrical characteristics of nerve preparations unsuitable for single fiber study and its extension to muscle experimentation seems feasible.

Glucose metabolism in marine Annelids. ELIZABETH SETON AND CHARLES G. WILBER.

Previously published evidence indicates that an increase in the environmental temperature of the Sipunculid, *Phascolosoma gouldii,* is accompanied by an increase in the amount of glucose in the coelomic fluid. Further work was done in an attempt to ascertain whether a similar pattern obtains in other marine worms. *Amphitrite ornata* was exposed to various temperatures for different periods of time and the coelomic fluid then removed and analyzed for glucose. In specimens exposed to 32° for times up to 12 hours, there is an appreciable increase in coelomic fluid glucose. If time in hours is plotted against concentration of glucose for a given temperature, a curve is obtained which rises from 32 mg. per 100 cc. (control value) and approaches asymptotically a maximum of 66 mg. per 100 cc. A similar curve plotted for Phascolosoma rises from the control value of 17 mg. per 100 cc. and approaches a maximum of 36 mg. per 100 cc. The calculated temperature coefficient for the process in Amphitrite varies between 1.3 and 2.0. Exposures to 38° results in a tremendous increase of fluid glucose in Amphitrite, and in first a marked decrease and then an increase of glucose in Phascolosoma. The origin of the glucose is not known at this time.

Effect of arsonoacetic, trans 1,2, cyclopentanedicarboxylic, and β-phosphonopropionic acids on enzyme systems in the ciliate, Colpidium campylum. GERALD R. SEAMAN AND ROBERT K. HOULIHAN.

Klotz and Tietze recently (*J. B. C.* **168**: 399) synthesized phosphonate, arsonate, and sulfonate analogues of succinic and malonic acids as well as cyclic analogues. Their results obtained with rat liver indicate that only the sulfonate analogues are capable of interacting strongly with succinic dehydrogenase. It therefore seemed desirable to test the efficiency of

some of these analogues using the ciliate protozoan, *Colpidium campylum.* The membrane of Colpidium is impermeable to succinate. Tissue homogenates were therefore used.

At inhibitor-substrate ratios of 1:2, β-phosphonopropionic, malonic, *trans* 1,2, cyclopentanedicarboxylic, and arsonoacetic acids cause inhibitions of 15, 19, 41, and 47 per cent, respectively, of the oxidation of succinate (0.02 M). At ratios of 2:1 the inhibitions are 58, 59, 85, and 98 per cent respectively. The increased degree of inhibition found when the inhibitor-substrate ratio is increased indicates that the inhibitions are competitive.

To obtain indications as to the specificity of the action of the compounds, pyruvate and acetate were used as substrates (in concentrations of 0.02 M), using living cells rather than homogenates. In inhibitor-substrate ratios of 2:1, malonate, phosphonate, and arsonate cause inhibition of acetate oxidation by 38, 57, and 100 per cent respectively. When pyruvate is the substrate, the inhibitions 51, 63, and 83 per cent, respectively. Therefore, the inhibitions are probably specific in their locus of action. Cyclopentanedicarboxylic acid, rather than inhibiting, accelerates the oxidation of pyruvate and acetate by 41 and 39 per cent respectively.

When living cells are incubated with succinate, succinate plus arsonate, malonate, or phosphonate, there is no increase in oxygen uptake due to the impermeability of the cell membrane to succinate. However, with cyclopentane and succinate, there is an increased oxygen uptake of 47 per cent. This seems to indicate that *trans* 1,2, cyclopentanedicarboxylic acid, in some manner, increases the permeability of the cell membrane of *Colpidium* and thus allows succinate to enter.

Electrical changes in crab nerve in relation to potassium movement.[1] ABRAHAM M. SHANES.[2]

The depolarizations of the leg nerves of *Libinia emarginata* during anoxia and during repetitive stimulation, as well as the repolarizations during recovery, are found to have certain similarities indicative of a common underlying process. Thus, the potential changes are augmented by veratrine and altered by differences in the volume of solution in contact with the fibers. These and other considerations (Shanes and Hopkins, 1948) suggest that the basis for the polarization fluctuations is alteration in the mechanism of potassium retention. In keeping with this the potassium lost per impulse computed from the potential changes with stimulation is of the same order as the available analytical figures.

For anoxia, however, the literature (Cowan, 1934) indicates no potassium loss. This has therefore been reexamined by flame spectrophotometric analyses[3] of ca. 1 cc. samples of sea water successively put in contact with the same set of 3 or 4 nerves for half hour intervals. Typically, 2 samples were taken first in oxygen, then 2 in nitrogen, and finally another 2 in oxygen again. In all cases a loss of ca. 20 μM per gm. wet weight per hr. occurred in oxygen whether or not glucose was present; anoxia doubled or trebled this leakage and 50 to 100 mm. glucose reduced the increment; return to oxygen either completely stopped the potassium escape or actually caused an absorption of potassium, the recovery being less striking when glucose was present.

These analytical data are in complete accord with electrical measurements carried on under the same experimental conditions. Thus, the marked depolarization during anoxia is appreciably reduced by glucose and the amplitude of the repolarization in oxygen in correspondingly less.

Replacement of 50 to 75 per cent of the sodium of an artificial sea water with choline markedly and reversibly reduces the degree of depolarization occurring with anoxia or stimulation; an equivalent amount of choline chloride has no such effect when the sodium content of the medium is unaltered. These and other experiments suggest that the potassium escape occurs by exchange with extracellular sodium, possibly through failure of a sodium exclusion mechanism. Further study is needed, however, and is presently in progress.

[1] Supported in part by research grants from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and from the American Philosophical Society.

[2] Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, D. C.

[3] Dr. George Marmont very generously permitted the use of a Beckman flame spectrophotometer.

The chloride content of frog muscle. W. D. SHENK.

There has been growing evidence that the Van Slyke method of chloride determination, a method which involves wet washing, is inadequate for muscle. Accordingly, an attempt has been made to determine the merits of the Parr bomb method for chloride determinations in whole muscle. The principle of the method consists in burning the muscle with sodium peroxide in a Parr bomb. In order to accomplish the burning of wet muscle, the charge, consisting of sodium peroxide, potassium nitrate and benzoic acid is first carefully mixed on a watch crystal. The ignition cup is then layered with this mixture, the muscle carefully centered in the cup and the remaining charge added and ignited. After ignition, the solid mass of residue is dissolved in warm distilled water and is rendered neutral with concentrated nitric acid; 5 ml. of excess acid is then added. After the addition of a controlled amount of silver nitrate the solution is boiled for one hour to coagulate the silver chloride, cooled to room temperature, filtered, washed twice, and the washings added to the filtrate. The filtrate is then titrated with 0.01 N ammonium thiocyanate until one drop causes a color change which persists for one minute at room temperature.

A marked difference was noted in comparing the values obtained by analyzing small pieces of the same muscle using the Parr bomb method in contrast with the Van Slyke technique. Analysis of wet muscle after acid digestion gave an average of 21.016 milliequivalents of chloride per kilogram, whereas the Parr bomb determinations gave an average of 39.4 milliequivalents per kilogram. Dry muscle gave 57.2 milliequivalents of chloride per kilogram with the Van Slyke method and 130.35 milliequivalents with the Parr bomb method.

It is believed that muscle must contain bound chloride which resists detection by customary analytical procedures.

The photosensitive pigment of the squid retina. ROBERT C. C. ST. GEORGE AND GEORGE WALD.[1]

The retina of the squid, *Loligo pealii,* contains a high concentration of retinene$_1$, a precursor for the synthesis of rhodopsin and a product of its bleaching in the vertebrate eye. In the squid retina, retinene$_1$ is found both free and bound to protein; and illumination of the dark adapted retina releases retinene$_1$. From these data it was concluded that the squid retina contains a photosensitive pigment closely related to rhodopsin, and like it capable of bleaching in the light to yield retinene$_1$ (Wald, *Amer. J. Physiol.* 133: 479, 1941; *Biol. Symp.*, VII, 43, 1942).

Recently Bliss has extracted a red pigment ("cephalopsin") from the squid retina which resembles rhodopsin in its visible absorption spectrum, and which yields retinene$_1$ on chemical destruction. Bliss suggests that this may be the visual pigment of the squid, but states that though it decomposes rapidly in the dark, it is not affected by light (*J. Biol. Chem.* 176: 563, 1948). It could not, therefore, be the source of the retinene$_1$ liberated by light from the retina; nor is it clear how a pigment that is not affected by light can be responsible for vision.

We have now examined the behavior of a pigment extracted from squid retinal rods by a procedure not materially different from that used by Bliss, and used by us earlier in the preparation of frog and cattle rhodopsin. Its absorption spectrum, like that of rhodopsin, consists of three bands: an α-band at 490 mμ, a low β-band at 365 mμ, and a high protein γ-band at 279 mμ. It is stable for many hours at room temperature in darkness. In the light it undergoes a photochemical change followed by a "dark" reaction, comparable with the transformation of rhodopsin to lumi- and to meta-rhodopsin (Wald, Durell and St. George, *Science*, in press). At temperatures above 23° C. these processes initiated by light are followed by bleaching in darkness to a mixture of retinene$_1$ and the regenerated photopigment in proportions roughly 3 : 1. Except for certain differences in the temperature characteristics of retinene$_1$ formation, there is no important distinction between this pigment and vertebrate rhodopsin. We propose therefore to refer to it simply as rhodopsin. It is the first light-sensitive pigment to be demonstrated directly in an invertebrate eye.

[1] This investigation was supported in part by a grant from the Medical Sciences Division of the Office of Naval Research.

An experimental method for rapid determination of extra-polar potential distribution in nerve. PETER A. STEWART.

Since slow changes leading eventually to the complete disintegration of a dissected nerve always occur, it is essential that data be taken rapidly from such preparations. Fortunately the data needed for studies of effective membrane impedance are well suited to rapid determination.

Simple circuit analysis indicates that under sub-threshold sinusoidal stimulation the effective conductance and susceptance of the membrane at any one frequency are determined by α, the exponential rate of decay of the amplitude, and β, the linear rate of phase change with distance of the extra-polar voltage. The susceptance is proportional to the product of these factors, the conductance to the difference of their squares, the proportionality factor being the longitudinal resistance per centimeter of the nerve.

To determine α and β, the nerve is mounted vertically in four ring electrodes in a moist chamber. One pair of electrodes is used as pick-up electrodes, one of which moves along the nerve; the other pair as stimulating electrodes. The voltage at the movable electrode is amplified and applied to the vertical deflection plates of an oscilloscope through a special exponentially tapered potentiometer. The rotation of this potentiometer drives a pen along the ordinate of a sheet of graph paper, which is moved laterally in synchronism with the motion along the nerve of the pick-up electrode. Turning the potentiometer to keep the amplitude of the oscilloscope pattern constant as the pick-up electrode moves yields a straight line plot of the logarithm of voltage against distance, the slope of which is α.

A sinusoidal voltage of variable phase is used as a horizontal deflection voltage on the oscilloscope. By varying this phase to keep the shape of the pattern constant, a pen driven by the phase control knob draws a straight line plot of phase against distance, the slope of which is β.

It is found to be possible to draw such a pair of curves in about 30 seconds.

The significance of the periblast in epiboly of the Fundulus egg. J. P. TRINKAUS.

The significance of the periblast layer in the early development of the Teleost egg has long been a matter of speculation. An experimental approach to the problem, however, is clearly to be desired. The present investigation constitutes such an approach. It is concerned with the role of the periblast in the morphogenetic movements of epiboly in the eggs of *Fundulus heteroclitus*.

Although the blastoderm as a whole clings rather closely to its underlying syncytial periblast, invaginated cells of the embryonic shield particularly tending to adhere to it, the only actual connection of the two layers is at the margin of the blastoderm. Here the blastoderm is connected to the periblast by means of its thin outer epiblast layer ("Deckschicht") and its accompanying surface gel layer. By severing this connection in blastula and gastrula stages, the periblast may be entirely freed of the blastoderm and its subsequent development observed.

(It may be noted, incidentally, that a technique is hereby available for culturing blastoderms which are truly yolk-free, as well as periblast-free. These would constitute excellent material for an analysis of the role of the yolk and periblast in embryonic differentiation.)

After removal of the blastoderm, such an exposed periblast will completely encompass the yolk sphere in an epibolic movement that proceeds at about the same rate as in normal controls. In the course of this epiboly the periblast layer becomes thinner and the nuclei more widely dispersed, as in the periblast of intact eggs. These facts and the results of carbon marking experiments on exposed periblasts indicate that there is a general expansion of the periblast in the course of epiboly.

When only the central area of the blastoderm is removed in late blastula or gastrula stages, and the marginal cells (e.g., germ ring) are left attached to the periblast, epiboly proceeds at close to normal rate. The margin of the blastoderm is thus carried vegetally resulting in a bunching of cells at "closure of the blastopore."

These experiments suggest that epiboly in the intact Fundulus egg is probably not due to an autonymous expansion of the blastoderm. Attention is directed rather to the activities of the periblast, whose epibolic tendencies may be an important factor in initiating and controlling the epiboly of the overlying blastoderm.

The behavior of the surface gel layer of the Fundulus egg during epiboly. J. P. TRINKAUS AND ROSEMARY GILMARTIN.

The behavior of the surface gel layer in epiboly was studied by the method of carbon marking, the fate of the adhering carbon particles being followed by means of camera lucida tracings. Eggs of *Fundulus heteroclitus* served as material for the investigation.

A carbon particle, adhering to the surface gel layer of the yolk sphere vegetal to the blastoderm, is approached during epiboly by the advancing margin of the blastoderm. When reached by the edge of the marginal periblast, it remains at this point and is carried vegetally during the continuing epiboly. A mark deliberately placed on the margin of the periblast behaves in a similar fashion. This is true for both intact eggs and eggs in which the periblast had previously been completely exposed by removal of the entire blastoderm (see Trinkaus, 1949, *Biol. Bull.*). A mark on the margin of the blastoderm itself adheres to this point during the course of epiboly in a similar fashion.

As the blastoderm envelops the yolk in epiboly, the surface gel layer of the yolk sphere continually decreases in area until with closure of the blastopore it disappears. A question arises, therefore, as to the fate of the surface gel layer of the yolk sphere. Does it contribute to the surface gel layer of the blastoderm and thus become underlain by the epiblast cells of the advancing blastoderm; or does it contribute to the surface of the expanding periblast? These marking experiments do not support either possibility. They suggest rather that the surface gel layer of the yolk sphere somehow disappears from the surface at or in the immediate vicinity of the margin of the periblast. This occurs in such a manner, however, as not to disrupt the connection between the surface of the periblast and the surface gel layer of the yolk. Exactly how this takes place is, of course, open to conjecture. These data, furthermore, support the conclusion that epiboly of both the cellular blastoderm and the underlying syncytial periblast entails an expression of material already present in these areas at the onset of gastrulation.

A preliminary study of the factors influencing the distribution of bottom fauna in two narrow arms of Buzzards Bay. GEORGE C. WHITELEY, JR., WILLIAM D. BURBANCK, AND MADELENE E. PIERCE.

Study of the bottom organisms of Rand's Harbor, selected for an experiment in fertilizing small arms of the sea, was carried out in 1946, 1948, 1949. The 1949 series of transects is the most complete one so far.

During 1946 when extensive salinity records were kept, salinities at the surface varied considerably from June to September, yet bottom salinities varied only from 27–29 parts per thousand. There is a large fresh water inflow to each arm and a tidal exchange [1] of at least 50 per cent of the total volume of the arms.

During 1949 in the large arm, 67 sampling stations along 8 transects were studied; in the small arm, 53 sampling stations along 7 transects were studied. All samples, except at high and low tide, were collected from a boat by means of a modified Ekman dredge 482 sq. cm. in area, or by a small orange peel dredge of approximately the same area.

Forty-three species of invertebrates including the locally rare *Cyathura carinata* were identified. Of these 11 genera, namely, Cistenides, Clymenella, Glycera, Neanthes, Haploscoloplos, Lumbrinereis, Mya, Mulinia, Nassa, Venus, and Uca, occurred in at least 50 per cent of the transects.

Distribution of the species most frequently occurring in 1949 was similar to that of 1948 and 1946, with the exception of *Cistenides gouldi* which was 200 times more numerous in 1948.

A comparison according to number of species found at different depth zones showed that the zones of low tide and slope not only supported the greatest diversity of species but also the largest number of individuals. Distribution according to substrate showed that sandy mud and sand and organic material were preferred substrates.

The mud snail, *Nassa obsoleta*, is the most characteristic animal of this harbor.

It is believed that variations in bottom temperatures and bottom salinities are among the least critical factors influencing distribution. Turbidity due to tidal and current action may interfere with normal feeding of the filter feeders.

[1] Data supplied by Albert Rosenburg.

The particle status of proteins. DOROTHY WRINCH.

The Sorensen view that many native proteins are systems of dissociable components has long been held. Today physicochemical evidence and evidence from the more precise studies in protein crystallography are accumulating which indicate that some even relatively small proteins are particles rather than single chemical entities.

The early x-ray studies of dry crystalline insulin (Crowfoot, *Chem. Rev.* **28**: 215, 1941) uncovered a trigonal structure of molecular weight ca. 36,000. Physicochemical investigations (Oncley) show that, at pH sufficiently acid, this structure dissociates into thirds and a preliminary x-ray study (Low, Cold Spring Harbor Sym. 1949) indicates that these subunits are identical. Thus the 36,000 structure may be pictured as 3 identical subunits on a 3-fold axis. The low pH required for dissociation suggests that the subunits are held together by hydrogen bond circuits of end groups on glutamine or asparagine substituents, after the manner of the acetamide crystal.

It has long been known that mild treatments, such as dilution, dissociate horse hemoglobin of molecular weight ca. 66,700 into 2 subunits and x-ray studies (Crowfoot, loc. cit.) indicate that these subunits are identical and are arranged on a 2-fold axis. Now the results of an x-ray study (Kendrew, *Acta Cryst.* 1, Dec. 1948) of horse and whale myoglobin, both with molecular weight ca. 16,700, raise the possibility that the subunits of horse hemoglobin may also be particles. It is interesting that a comprehensive amino acid analysis of whale myoglobin (Schmid, *Nature* **153**: 481, 1949) puts the residue number at 147, close to one quarter of 576, the number suggested for the hemoglobins (Wrinch, *Biol. Bull.* **95**: 247, 1948). It may be that the hemoglobins comprise 8 subunits of ca. 72 residues a piece, or 12 subunits of ca. 48 residues a piece, with the myoglobins comprising 2, or 3, such subunits. These would be compatible with C_1 skeletons, with 8 hexapeptides on t^+ faces, 8 also on t^- faces and 6 tetrapeptides on cube faces, or with one set of hexapeptides or the set of tetrapeptides deleted (Wrinch, *Science* **107**: 445, 1948). The finding that there is one free (valyl) α-NH_2 in whale myoglobin (Schmid), one free (glycyl) α-NH_2 in horse myoglobin, 6 free (valyl) α-NH_2 in horse hemoglobin (Porter and Sanger, *Biochem. Journ.* 42: 287, 1948) does not imply that the structures are 1, or 6, "chains," but only that there are 1, or 6, residues or peptides functioning as substituents, locked into the skeletons by a single terminal (Wrinch, *Wallerstein Communications* **11**: 175, 1948).

Even these two examples show the crucial issues raised by a particle as opposed to a molecular status for proteins.

So far there seems no escape from the view that the protein essence resides in a few, maybe only a single, characteristic type of skeleton, which is stabilized by associations of the substituents emerging therefrom. The finding that "proteins" occur in a vast variety of sizes and shapes does not conflict with this view, once it is interpreted as in general referring to particles and not to molecules.

With the specificity of individual proteins located in the nature, arrangement and association of the R-groups, a particle will have a specificity dependent on the spatial pattern of its molecules. This means that mild treatments, leading to gross changes in size and shape, may effect changes in biological specificity. Such phenomena are to be distinguished from the entirely different type of change in a protein, often called "denaturation," which on the present view result from disjoining of the skeleton and loss of protein status.

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Experiment studies on the heat regulation arctic and tropical warm blooded animals. P. F. SCHOLANDER. No abstract submitted.

The uptake and loss of K^{42} in the unfertilized and fertilized eggs of Strongylocentrotus purpuratus and Arbacia punctulata. EDWARD L. CHAMBERS.[1]

Carrier free K^{42} was added to 0.2 per cent suspensions of unfertilized and fertilized eggs and the penetration of K^{42} into the eggs measured by the method already described (*Biol. Bull.* **95**: 252 and 262, 1948). After a sufficient quantity of K^{42} had entered the unfertilized eggs, a

[1] Under grant from the NCI, USPHS. University of California at Berkeley, New York University, and the Eli Lilly Research Laboratories, M.B.L., Woods Hole, Mass.

portion of the suspension was removed, the eggs centrifuged down and washed twice with sea water. Two suspensions of these washed eggs were prepared and one lot fertilized. The loss of K^{42} from these washed eggs was measured by removing samples at intervals of time and determining the radioactivity remaining in the eggs.

In the uptake experiments the specific activity of the fertilized eggs (a_i/C_i) reaches 85 to 100 per cent of the specific activity of the surrounding sea water (a_0/C_0) after a period of 15 to 20 hours. The specific activity of the unfertilized eggs slowly rises to 20 per cent of the sea water after 15 hours. After this time the eggs lose their fertilizability.

The fraction of total in the eggs exchanged per minute (k) was calculated from the K^{42} uptake data using the following equation, adapted from Krogh, 1946:

$$k_{(\text{uptake})} = \frac{1}{t} \ln \frac{1}{\left(1 - \frac{a_i}{a_0}\frac{c_0}{c_i}\right)},$$

and from the K^{42} loss data, using the equation:

$$k_{(\text{loss})} = \frac{1}{t} \ln \frac{1}{(a_i/a_{i_0})}.$$

In these equations, t = time in minutes after adding K^{42} to the suspension in the case of the uptake experiments, or after washing the eggs free of K^{42} in the loss experiments, $a_i[K^{42}]$/ml. eggs, $a_{i_0}[K^{42}]$/ml. eggs at 0 time, C_i mM K^{39}/ml. eggs, C_0 mM K^{39}/ml. sea water.

The plots of $\ln\left(1 - \frac{a_i}{a_0} \cdot \frac{c_0}{c_i}\right)$ and of $\ln\left(\frac{a_i}{a_{i_0}}\right)$ against t are linear. Very dilute suspensions of eggs were used in order that the above simplified equations could be used. The results obtained in two typical experiments on the eggs of *S. purpuratus* are presented in the accompanying table:

	$k_{(\text{uptake})}$	$k_{(\text{loss})}$
Unfertilized	.00022	.00053
Fertilized	.035	.083
$\frac{k_{(\text{fertilized})}}{k_{(\text{unfertilized})}}$	16	16

The fraction of total K exchanged per minute calculated from the data on the loss of K^{42} was approximately twice that calculated from the data on the uptake of K^{42} (see table). This indicates that all the K within the egg does not exchange with the exterior at the same rate. However, on the basis of the calculations of both the uptake and loss experiments, the $k_{(\text{fertilized})}$ values are 16 times the $k_{(\text{unfertilized})}$ values (see table). The overall rate of K exchange in the fertilized eggs is, therefore, 16 times faster than in the unfertilized eggs.

The glycogen content of some invertebrate nerves. WILLIAM SCHALLEK.

Holmes (*Biochem. J.* 23: 1182, 1929) observed that nerves of the crab Cancer had a glycogen concentration many times greater than that of mammalian nerve. It seemed desirable to repeat and extend these observations, using improved methods of glycogen assay (Kerr, *J. Biol. Chem.* 116: 1, 1936). Glycogen and galacto-lipids were removed from the tissue with alcoholic KOH; the lipids were then removed with a methyl alcohol-chloroform mixture. The glycogen was hydrolyzed in HCl; the resultant glucose was assayed with a photo-electric colorimeter, using the copper reagent of Somogyi (*ibid.* 160: 61, 1945) and the color reagent of Nelson (*ibid.* 153: 375, 1944). The data is presented as mg. glycogen per 100 g. fresh weight of tissue. A and B are separate experiments, performed on different animals on different days.

	A	B	
Spider crab, *Libinia emarginata*			
Leg nerve	1680	1545	mg. per cent
Ganglion	1480	1180	

	A	B	
Squid, *Loligo pealei*			
Stellate ganglion	463	436	mg. per cent
Optic ganglion	259	236	
Cerebral ganglion	232	226	
Stellar nerve	58.5	39.8	
Axoplasm	<1	<1	
Remainder of nerve	21.7	20.1	

For comparison, the following data on mammalian nerve may be quoted: Rabbit nerve, 48 mg. per cent, Holmes et al., *Am. J. Physiol.* **93**: 342, 1930. Rabbit brain, 82 mg. per cent, Kerr and Ghantus, *J. Biol. Chem.* **116**: 9, 1936.

The amount of glycogen in the squid ganglia is intermediate in value between that of rabbit brain and the crab ganglion. Squid nerve, on the other hand, has practically the same glycogen content as rabbit nerve; crab nerve differs markedly in having a concentration approximately 30 times as great.

The axoplasm was separated from the remainder of the squid nerve by extrusion into distilled water. Here it formed a cylindrical clot, which was promptly transferred to KOH. No detectable glycogen was found in the axoplasm; all the content was in the remainder of the nerve. The concentration in the remainder should therefore be greater than that in whole nerve; this does not appear in the data, apparently because the determinations on whole nerve were made on different animals some time before the determinations in which the axoplasm was extruded. The distribution of glycogen outside the axoplasm accords with that reported for cholinesterase and adenosinetriphosphatase, but not with that for succinic dehydrogenase and cytochrome oxidase (Nachmansohn et al., *J. Neurophysiol.* **4**: 348, 1941; **5**: 109, 1942; **6**: 203, 1943; Libet, *Biol. Bull.* **95**: 277, 1948). Havet has reported that glycogen is concentrated in the neuroglia rather than in the neurons (*Cellule* **46**: 179, 1937).

PAPERS READ BY TITLE

Description of and lipid localization in cells of body cavity fluid of Arenicola marina (Lamarck) and Amphitrite ornata (Leidy). Ruth P. Alscher.

Romieu (*Archives de Morphologie Générale et Expérimentale* **17**: 1923) described the cells found in the body cavity fluid of *Amphitrite Edwardii, Amphitrite rubra* and some of the cells from *Arenicola marina.*

More detailed studies of the cells in the body cavity fluid of *Arenicola marina* and another species, *Amphitrite ornata,* were made employing modern techniques. The body cavity fluid from *Arenicola marina* and *Amphitrite ornata* was obtained with the aid of a hypodermic needle and syringe. Microscopical observations of various samples of fresh body cavity fluids of these marine annelids were made.

It was observed that the fluid from *Arenicola marina* contains 5 different types of cells of which 2 types are sexual cells: (1) small, mononucleated cells with heterogeneously granular cytoplasm; (2) large, mononucleated cells with lobes of densely granular cytoplasm; (3) small, amoeboid cells; (4) egg cells; (5) sperm cells.

The body cavity fluid from *Amphitrite ornata* contains 8 different types of cells of which 2 types are sexual cells: (1) large, anucleated cells with red pigment granules; (2) large, oval, mononucleated cells with heterogeneously granular cytoplasm; (3) small, oval, anucleated cells with densely granular cytoplasm; (4) small, anucleated cells with clear cytoplasm; (5) small, mononucleated cells with yellow, homogeneous cytoplasm; (6) large, anucleated, vacuolar cells; (7) egg cells; (8) sperm cells.

Measurements of 50 cells of each type were made. See Table I.

The localization of lipids in these cells was studied from smear preparations of the body cavity fluids, and both the Telford Govan (*J. Path. Bact.* **56**: 262–264, 1944) and Jackson (*Onderstepoort J. Vet. Sci. Animal Ind.* **19**: 169–177, 1944) procedures for the testing of lipids with slight modifications in technique were made. It was observed that Jackson's procedure

TABLE I

Species	Types of cells	Length of cells	Width of cells	Number of droplets of lipids per cell
Arenicola marina	1. Small, mononulceated cells	11.55μ–26.4μ	11.55μ–26.4μ	4–12
	2. Large, mononucleated cells	23.1μ–49.5μ	23.1μ–46.2μ	21–28
	3. Small, amoeboid cells	6.6μ–23.1μ	4.95μ–13.2μ	2–5
	4. Egg cells	56.1μ–231μ	56.1μ–231μ	14–43
	5. Sperm cells	The cells are visible, but they are too small to measure accurately		1–2
Amphitrite ornata	1. Large, anucleated cells with red pigment	42.9μ–121.55μ	42.9μ–121.55μ	4–38
	2. Large, oval, mononucleated cells	24.75μ–49.5μ	13.2μ–33μ	4–29
	3. Small, oval, anucleated cells with densely granular cytoplasm	13.2μ–18.15μ	6.6μ–18.15μ	1–10
	4. Small, anucleated cells with clear cytoplasm	11.55μ–82.5μ	11.55μ–74.25μ	2–3
	5. Small, oval, mononucleated cells with yellow, homogeneous cytoplasm	9.9μ–33μ	8.25μ–33μ	2–26
	6. Large, anucleated, vacuolar cells	28.05μ–33μ	23.1μ–33μ	21
	7. Egg cells	128.7μ–269.8μ	114.4μ–255.6μ	19–92

was more satisfactory for these tissues because the lipids were stained a more brilliant and clearer red.

With the aid of camera lucida drawings the size and distribution of the lipid globules were studied. In most cells, the size of the droplets of lipids ranged from small to very large. The droplets of lipids were located at various depths throughout the cytoplasm of all the cells. An interesting fact to be noted is that the number of droplets of lipids varied in the different types of cells from the 2 worms (Table I). The egg cells in both species have the greatest number of droplets of lipids, and the larger cells have more droplets of lipids than do the smaller cells. This indicates that a possible correlation between cell size and lipid content can be made, i.e., the larger the cell the greater is the lipid content.

Detection of physiological mutants in Neurospora without the use of selective media.[1] K. C. ATWOOD.

In Neurospora the mycelia of two different mutants, neither of which can grow on minimal medium, will readily fuse to give a mycelium which grows on minimal medium and contains a mixture of the nuclei of the two mutant strains in a common cytoplasm. Such a heterokaryon

[1] This work was supported in part by a grant from the American Cancer Society administered by Prof. Francis J. Ryan of Columbia University.

can grow on minimal medium because the nuclei of each mutant component can perform the function which cannot be carried out by nuclei of the other component.

This system has been adapted to the detection of new mutants as follows: A heterokaryon is made between any biochemical mutant, X-less, and a morphological mutant, No. 422. By itself No. 422 grows as a mass of coherent spheres, and X-less will not grow unless substance X is provided. The heterokaryon, however, has normal morphology, and grows on minimal medium. Macroconidia having several nuclei per conidium are produced by the heterokaryon, and three classes of conidia can be distinguished by plating, using the sorbose technique. Those conidia containing all X-less nuclei give rise to colonies of normal morphology which appear only if substance X is present, and those containing only No. 422 nuclei give colonies having the morphology of the No. 422 mutant. Those containing both X-less and No. 422 nuclei give normal colonies appearing on minimal plates, and when conidia of cultures established from this type of colony are plated, the three classes may be distinguished as before. However, if such colonies are isolated following treatment of the conidia with a mutagenic agent, and the conidia of the resulting cultures plated again on minimal medium, some of the plates show no colonies with No. 422 morphology. In these cases, the conidia containing only No. 422 nuclei have failed to form colonies because in some the treated conidia which contained nuclei of both types, mutations were induced in the No. 422 nuclei. A mutation occurring here, while it does not affect the growth of the heterokaryotic culture arising from a treated conidium, precludes growth on minimal medium of the No. 422 component when nuclei of this component are isolated from the other nuclei during the formation of conidia by the heterokaryotic culture.

With this method the variety of mutants obtained is not restricted by the usual limitations of selective media, since the medium which supports the growth of the mutant nuclei is the living cytoplasm itself.

Ribonucleinase activity in the development of the sea urchin, Arbacia punctulata. MAURICE H. BERNSTEIN.

The behavior of RNA during development has been elaborately described. This behavior must depend on underlying enzyme mechanisms. It was, therefore, considered important to examine the level of ribonucleinase activity during the early period of development of the sea urchin.

Estimations of activity in the eggs and the early embryos of *Arbacia punctulata* were made according to the manometric methods described by Bain and Rusch (*J. B. C.* 153: 659, 1944). Eggs or embryos were homogenized in a glass homogenizer and put into a bicarbonate medium containing RNA as a substrate. The evolution of CO_2 was followed manometrically in a conventional Warburg apparatus. This method is based on the known splitting action of the enzyme on RNA; the acid groups liberated by this action drive off CO_2 from the bicarbonate medium; hence, CO_2 liberation is a measure of the enzyme activity.

A sharp drop in the activity of ribonucleinase was found at or shortly after fertilization. This lower level of activity was then maintained during the first twenty hours of development, which was as long as any of the embryos were cultured. Schmidt, Hecht, and Thannhauser (*J. G. P.*, 31: 203, 1948) found the DNA content of Arbacia eggs rose sharply during the first twenty-four hours of development, while the RNA content remained constant. Mazia et al. (*Biol. Bull.* 95: 250, 1948) have shown that the level of desoxyribonuclease activity does not change in this early period. These and other data support the idea that RNA is not a precursor of DNA.

Effect of redox dyes on development of marine eggs. MATILDA M. BROOKS.

These investigations are a continuation of studies on the mechanism of fertilization of marine eggs as related to redox potential. The methods used are described in a previous paper.[1] *Chaetopterus pergamentacea* and *Amphitrite ornata* were used. Unfertilized eggs were placed in sea water containing 2×10^{-5} per cent of one of the series of redox dyes for varying periods of time from ½ minute to 6 hours, and then transferred to sea water alone. The redox

[1] M. M. Brooks, *Growth* X, 391, 1946.

potential of the unfertilized eggs and sperm, respectively, were measured on the Coleman electrometer as previously described.

It has been shown by Lillie [1] and others that these eggs form irregular development as amoeboid forms apparently spontaneously. A few such forms were always found in the controls in spite of careful handling. However, when the eggs were subjected to a dye low in the redox scale, such as neutral red, there was about 90 per cent development in Chaetopterus including two many-celled stages, the majority as amoeboid forms with or without membranes and several swimming blastulae, which did not develop further.

In the case of Amphitrite, amoeboid forms were always in the controls, but at least three times as many when indigo tetra sulfonate dye in sea water was used.

In these preliminary experiments, it shows again the influence of an appropriate redox potential in causing development in marine eggs.

Inactivation of Cypridina luciferase by heat. AURIN M. CHASE.

Luciferase solutions from the crustacean, *Cypridina hilgendorfii,* were subjected to temperatures from 40° to 60° C. for times up to 24 hours. After the desired exposures samples were cooled rapidly to 25°, luciferin (purified by Anderson's procedure; *J. Gen. Physiol.*, 1935) was added and the luminescent reaction measured. The resulting first order velocity constants were expressed in terms of that for the reaction catalyzed by untreated enzyme.

Above 50° luciferase activity practically disappears in 15 minutes. Between 40° and 50° a rapid initial activity loss occurs during about one hour. The rate of loss then decreases abruptly, remaining relatively low. The equation for a simple first order reaction does not describe the data; rather, a mechanism involving at least two steps is indicated.

Upon cooling enzyme solutions to 25° after 15 minutes at 48°, about half of the lost activity is regained asymptotically during four hours indicating a reversible effect of temperature.

The experiments for temperatures from 40° to 50° can be described by an equation based on the following assumed mechanism:

$$N \text{ (active)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I_1 \text{ (inactive)} \xrightarrow{k_3} I_2 \text{ (inactive)}.$$

N represents the original native form of the luciferase. I_1 is an unstable inactive form. I_2, irreversibly produced from I_1, is also inactive. k_1, k_2 and k_3 represent the velocity constants of the reactions indicated. Experiments with certain other enzyme systems have indicated a similar mechanism (e.g., Herriott, *J. Gen. Physiol.*, 1948; Kunitz, *ibid.*, 1947).

For luciferase, analysis shows k_1 and k_2 to be about 100 times as great as k_3. k_1 increases with temperature to yield an experimental activation energy of about 50,000 calories indicating protein denaturation. The corresponding value for k_3 is about 30,000. k_2 is apparently independent of the temperature, at least from 40° to 50°, but this result may be illusory.

Gross morphological effects of low temperature on the fertilized eggs of Chaetopterus. DONALD P. COSTELLO AND CATHERINE HENLEY.[2]

At the time of insemination the normal Chaetopterus egg is at the metaphase of the first maturation division. In order to study the effects of low temperature on the processes of maturation and early cleavage, experiments have been conducted involving exposure of fertilized eggs to temperatures of 2 to 5 degrees C. beginning immediately after insemination. The duration of treatment ranged from 30 minutes to 12 hours, but the observed effects were most striking after a treatment of 5 hours or longer.

The process of polar-body extrusion is completely inhibited in these cold-treated eggs. With the gradual rise to room temperature, development is resumed; the eggs round up and lose the irregular flattened shape which is observed during the exposure to cold. Within 30 minutes after the end of the treatment, there is a very exaggerated elevation of the fertilization

[1] F. R. Lillie, *Archiv f. Entwickelungsmechanik der Organismen* XIV, 477, 1902.

[2] Aided by grants from the American Philosophical Society and the Carnegie Research Fund of the University of North Carolina.

membrane (normally quite inconspicuous in the Chaetopterus egg), similar to that observed in the egg of Nereis after treatment with alkaline sodium chloride (pH 10.5). Treatment with alkali does not produce accentuation of membrane elevation in normal fertilized Chaetopterus eggs.

Cleavage begins shortly after membrane elevation; the "pear" and polar lobe stages characteristic of the normal egg are not usually recognizable. The daughter-cells resulting from the first cleavage are often equal in size; subsequent cleavages are usually very irregular and difficult to study in living material. Numerous small blebs occur over the surface of the egg before and during cleavage, and these blebs may be so pronounced as to simulate cytolysis. However, the division process continues and very atypical larvae result. No readily identifiable double embryos were observed, but exceptionally large larvae (presumably fusion products) and exogastrulae occur frequently.

Cytological studies in Nymphaea L. A. ORVILLE DAHL.

Flower bud material of the white water lily (*Nymphaea odorata* Ait.) was collected at Hyannis, Mass., for an ontogenetic analysis of its highly distinctive ring-furrowed pollen grain. Material was preserved in 1/3 propionic acid-ethanol and in Karpechenko's variation of Navashin's fixative. The meiotic condition of the anthers of various ages was determined from aceto-carmine preparations. This survey indicates that the colony of plants is of interest in deviating in its chromosome number from that already published for this species. A gametic number of 42 was reported by Langlet in 1927 on the basis of other material. The collection from Hyannis has a gametic number of 28. Within the genus this number has been observed only in the new world *N. mexicana* Zucc. and one old world species (*N. Lotus* L., including "N. rubra Roxb."). At metaphase and late anaphase II (polar views), the length of the chromosomes is somewhat less than 1 micron. Starch grains are so abundant in the cytoplasm that observation of chromosomes and spindle substance is frequently rendered difficult. This was particularly the case in division II of meiosis where the relatively small spindles are largely enveloped in starch.

A larger sampling of the species throughout its range is needed for proper evaluation of any differentiation in the constitution of the chromosomal complement.

Fertilizin from the eggs of the clam, Mactra solidissima. CHARLES B. METZ AND JOANNE E. DONOVAN.[1]

The view that fertilizin plays a significant role in fertilization has been questioned on the grounds that fertilizin has not been shown to occur universally. Therefore, it is of interest to report the presence of this sperm isoagglutinin in the egg water (supernatant sea water of egg suspensions) of another form, the clam *Mactra solidissima.* Mactra fertilizin was first detected by its effect on the fertilizing power of homologous sperm: Mactra egg water markedly reduced the fertilizing power of Mactra sperm. This inhibitory effect was ascribed to fertilizin because Mactra blood lacked this property. Since no agglutination of homologous sperm by Mactra egg water was observed in early tests, it was at first assumed that natural Mactra fertilizin existed only in a non-agglutinating univalent form. More recently, however, strong fertilizin preparations have been obtained which have pronounced agglutinating action on Mactra sperm. Therefore Mactra fertilizin can exist in multivalent form. Strong fertilizin preparations appeared to have a moderate activating as well as agglutinating effect on homologous sperm. The sperm agglutinated tail to tail, head to head and probably head to tail. The agglutination did not reverse appreciably on standing for one hour. Mactra fertilizin was specific to the extent that it did not agglutinate Echinarachnius or Arbacia sperm.

Adenosine triphosphate and the luminescence of the "railroad worm" and other luminous organisms. E. NEWTON HARVEY.

McElroy's (*Proc. Nat. Ac. Dc.* 33: 342, 1947) demonstration of the striking action of adenosine triphosphate (ATP) in continuing the luminescence of extracts of fire-fly lanterns has raised the question as to how general the ATP action is on other groups of luminous animals. Negative results have been obtained by Chase and McElroy with extracts of the dried ostracod

[1] Aided by a grant from the National Institute of Health, U. S. Public Health Service.

crustacean, Cypridina, and by McElroy on luminous bacterial extracts. Recently Dr. P. Sawaya of São Paulo, Brazil, has sent me a specimen of the rare luminous beetle, Phrixothrix or "railroad worm," making it possible to test ATP on both red and yellow luminous organs. The ATP solution was prepared in the usual manner and shown to be active with a fire-fly (Photinus) lantern extract. No red luminiscence was obtained on adding ATP to a water extract of the red luminous organ of Phrixothrix and also none with the yellow luminescent organ. The latter result is not regarded as conclusive because the yellow light organs are very small and cannot be removed without much nonluminous tissue, thus making the extract highly dilute. The red light organ extract was concentrated and the experiment appears satisfactory. No luminescence was obtained on adding ATP solution to extracts of the luminous ctenophore, Mnemiopsis, or to extracts of the luminous annelid, Chaetopterus, under conditions regarded as satisfactory for demonstrating an ATP effect.

Cytological effects of low temperature on the fertilized eggs of Chaetopterus. CATHERINE HENLEY AND DONALD P. COSTELLO.[1]

A preliminary cytological study has been made of fertilized Chaetopterus eggs treated with low temperature, as described in the preceding abstract. Whole-mount Feulgen preparations were used chiefly; these were stained according to a method described by Anna R. Whiting (in press). A few additional preparations were made by the flattening method (Tyler, 1946). Treated and control eggs were fixed in Kahle's and Bouin's fluids at various intervals in order to secure samples of as many division stages as possible.

Polar bodies were not present in the great majority of treated eggs; at the end of the treatment 9 tetrads were clearly visible on each maturation spindle. These tetrad configurations were considerably more diffuse and complex than the normal ones.

A wide variety of abnormal mitotic configurations was observed in preparations fixed at the time of first and later cleavages in the treated eggs. These abnormalities included multipolar spindles, anaphase and telophase spindles with lagging and "lost" chromosomes or chromosome fragments, and masses of relatively uncondensed chromatin which apparently represented abortive prophase figures. In general, the types of mitotic anomalies observed were strikingly similar to those reported as occurring in the epithelium of larval salamanders exposed to low temperature. Multipolar figures were observed in a few of the control eggs; these are presumed to result from polyspermy. Since more than one sperm nucleus is often visible in the cold-treated eggs, the multipolar spindles in the experimental eggs may be the result of polyspermy facilitated by the cold treatment, rather than a direct effect of the low temperature.

Although extensive chromosome counts have not yet been completed, the available evidence indicates that at least some of these cold-treated eggs may be polyploid. Further cytological studies are in progress.

Studies of the Nereis egg jelly with the polarization microscope. SHINYA INOUÉ.

Beneath the vitelline membrane of the *Nereis limbata* egg, there is a layer of granules which disappear upon insemination and give rise to a very thick layer of jelly around the egg. Each of these granules possesses a strong birefringence with definite axis which suggest their crystalline nature. These granules are arranged radially and are partially responsible for the radial positive birefringence of the layer in which they are distributed.

The jelly extruded from the activated egg shows a radial positive birefringence which is stronger near the egg surface. This changes into a radial negative birefringence when the jelly is compressed. Also, a shearing force induces an apparently positive birefringence in the direction parallel to the shear. The induced birefringence in both cases is stronger, the stronger the deforming force.

If the egg is treated with saturated magnesium sulphate or ammonium sulphate and subsequently returned to sea water, or treated with distilled water, the vitelline membrane is elevated and a large clear zone appears. This zone shows a radial positive birefringence which is stronger towards the central protoplasmic mass. When such an egg is compressed to different

[1] Aided by grants from the American Philosophical Society and the Carnegie Research Fund of the University of North Carolina.

degrees, it shows proportionally strong radial negative birefringence, which returns to its original form in a few seconds after the deforming force is released. This indicates that the clear zone is filled with a gel of similar optical property as the extruded jelly.

Ferry has pointed out that this jelly is a polysaccharide, and the observation above also shows that the jelly is most likely composed of a linear polymer which has a larger index of refraction along the axis of its main chain.

A Feulgen-positive reaction of the oil droplets in the Nereis egg. ROBERTA LOVELACE.[1]

The surface of the oil droplets in inseminated eggs of *Nereis limbata* reacts positively to the Feulgen reagents when the eggs are fixed in Bouin's fluid or in Allen's B-15. Washing the eggs overnight in 95 per cent alcohol removes the Feulgen-positive substances from the oil-protoplasm interface in most eggs. Eggs which have been treated with isotonic calcium chloride at 5 minutes after insemination do not lose the positively-reacting substances readily in 95 per cent alcohol. When eggs are fixed in Kahle's fluid or in Carnoy-Lebrun fixative, the positive reaction of the oil drops is less conspicuous and may be absent in some eggs. In uninseminated eggs, the oil droplets are not affected by the Feulgen reagents, even when the fixing agent contains chromic acid. The oil droplets of developing embryos and of swimming trochophores also react positively following fixation in Allen's B-15, Bouin's and Kahle's fluids. Oil droplets are unaffected by the stain in non-hydrolyzed eggs. These observations suggest that some metabolic activity which is set off in the egg by contact with the sperm results in the formation of plasmal substances at the oil-protoplasm interface. For whole mounts of Nereis eggs and embryos (using a method devised by Anna R. Whiting), fixation for 1 hour in a mixture of 8 parts of Allen's B-15 stock solution with 2 parts of 10 per cent chromic acid solution gives beautiful preparations. A sulfur dioxide water bath before staining with leuco-basic fuchsin improves the preparations.

Sulfhydryl inhibitors and a seminal fluid factor in sperm respiration. LEONARD NELSON.

It has been previously observed that sulfhydryl inhibitors in low concentrations cause increases in oxygen consumption of sea urchin spermatozoa of up to 300 per cent. Sperm used in these studies had been centrifuged once and resuspended in filtered sea water. Under these conditions, 1×10^{-4} M cadmium chloride caused a 140 per cent increase and 1×10^{-3} M $CdCl_2$ caused a 51 per cent increase in oxygen uptake. Higher concentrations inhibited respiration. However, if the "dry" sperm is centrifuged for fifteen minutes at a relative centrifugal force of 1250, the seminal fluid decanted, the sperm washed twice with filtered sea water, and finally resuspended in the first wash water, the increase in oxygen uptake in the presence of $CdCl_2$ is halved (Table I); and if the spermatozoa are resuspended in diluted seminal fluid the cadmium effect is not noted.

The QO_2 of higher densities of sperm suspension in undiluted seminal fluid (conditions approximating those within the testis) is somewhat lower than that of the sperm in sea water.

TABLE I

Sperm density	Suspending medium	$CdCl_2$ (M)	O_2 uptake (CMM)	Per cent increase
			60 min.	
5 per cent	Filtered sea water	—	34	—
5 per cent	Filtered sea water	5×10^{-5}	50	47
5 per cent	First wash water	—	37	9
5 per cent	First wash water	5×10^{-5}	41	21
5 per cent	Diluted sem. fluid	—	63	85
5 per cent	Diluted sem. fluid	5×10^{-5}	63.5	87

[1] Aided by a grant from the Faculty Research Fund of the University of South Carolina.

TABLE II

Sperm density	Suspending medium	$CdCl_2$ (M)	O_2 uptake (CMM)	Per cent increase
			60 min.	
9 per cent	Filtered sea water	—	64	—
9 per cent	Filtered sea water	10^{-3}	104	63
9 per cent	Undiluted sem. fl.	—	56	−12.5
9 per cent	Undiluted sem. fl.	10^{-3}	87	36
9 per cent	Boiled sem. fluid	—	78	22
9 per cent	Boiled sem. fluid	10^{-3}	115	80
			120 min.	
5 per cent	Filtered sea water	—	81	—
5 per cent	Filtered sea water	10^{-3}	127	57
5 per cent	Ground glass sem. fl.	—	181	124
5 per cent	Ground glass sem. fl.	10^{-3}	140	72

Addition of $CdCl_2$ to the seminal fluid, boiling or filtering the fluid through ground glass, and even dilution in sea water, will raise the QO_2.

These data indicate the presence in seminal fluid of a factor perhaps similar to that described by Hayashi.[1] If this seminal fluid factor is likewise protein in nature, perhaps the stimulatory effect may be attributed to the inactivation of the factor by mercaptide-formation of its sulfhydryl groups by cadmium ions.

Extrusion of jelly by eggs of Nereis limbata under electrical stimulus. W. J. V. OSTERHOUT.

Insemination causes Nereis eggs to extrude jelly. This is produced by the swelling of jelly precursor granules inside the protoplasm. In the absence of sperm extrusion of jelly can be produced by weak electrical currents, e.g., by alternating or direct currents of 17 milliamperes per $cm.^2$ of cross section applied for 1 minute (W. Cattell obtained artificial parthenogenesis with much larger direct currents).

These experiments were made at approximately 15°, 20°, and 25° C.

When 17 milliamperes are applied for 1 minute the production of jelly continues for several minutes after the current is shut off. It is much more rapid with alternating than with direct current.

In direct current the jelly may appear first inside the vitelline membrane which covers the egg on the side facing the anode and then pass through the membrane into the sea water and more toward the anode.

In alternating current the jelly usually appears first on the opposite sides facing the electrodes, and extends gradually around the egg.

The action of electrical currents is readily blocked by treating the eggs with certain reagents.

These and other experiments indicate that the effects described are not due to heat developed by the current.

It may be suggested that the micelles of the jelly precursor granules migrate cataphoretically so as to promote access of water to the substance which swells to produce the jelly. If the granules have a waterproof covering as suggested by D. P. Costello this hypothesis may apply to the covering.

The influence of glycolysis on the potassium and sodium content of Saccharomyces cerevisiae and the egg of Arbacia punctulata. GEORGE T. SCOTT AND MARY E. RICE.

Danowski (1940) has observed a loss of potassium from erythrocytes in the presence of sodium fluoride. Harris (1941) in addition demonstrated a greater loss of potassium in the

[1] Hayashi. *Biol. Bull.* 89: 162, 195; 90: 177, 1946.

cold in the absence of glucose. Wilbrandt (1940) reported a loss of potassium in the presence of iodoacetic acid. Recently Dixon (1949) has indicated a significant potassium loss from brain slices under conditions of anaerobiosis in the absence of glucose or under conditions of fluoride inhibition.

The present experiments consist of a study of the relation of glycolysis to the potassium and sodium distributions in the Arbacia egg and the yeast cell. The cell suspensions are placed in 100 ml. graduated conical centrifuge tubes for the duration of the experiment. The cells are then thrown down by centrifuging, and the total cell volume measured. After a dry weight is taken the material is "ashed" by boiling in distilled water for twenty minutes to release the potassium and sodium. The solution is made up to volume, cell debris removed by centrifuging, and analyzed in the flame photometer.

Under conditions of anaerobiosis, indicated by decolorization of methylene blue, for short periods of time (one to three hours) the potassium and sodium content does not change. In the presence of iodoacetate (1 : 5,000) from 25 to 40 per cent of the potassium content is lost from the cells. A systematic study of the rate of change of these two elements over a longer period of time in the presence of inhibitors is in progress. The possible prevention of fluoride and iodoacetate inhibition by triose will be investigated.

The adaptive utilization of sucrose by the ciliate, Colpidium camplum. GERALD R. SEAMAN AND BENEDICT O'MALLEY.

A pure, sterile culture of *Colpidium campylum* was established in a medium consisting of 0.1M sucrose in Hahnert's solution by transferring 0.5 cc. of inoculum from a proteose-peptone medium to 10 cc. of the sucrose medium. When maximum growth was reached, 0.5 cc. of this culture was transferred to a second 10 cc. of sucrose medium. The procedure was repeated through 20 transfers. At that time calculations show that less than 1×10^{-5} micrograms of material had been carried over from the original peptone culture. All results presented were obtained from cultures which had undergone more than 20 transfers in the sucrose medium.

The growth rate in sucrose is very much lower than that obtained in peptone medium (Seaman, *J. Cell. Comp. Physiol.* 33: 137). A maximum population of 16,000 organisms/cc. is reached 3 days after inoculation. The number of cells then decreases rapidly to a level of approximately 4,000 organisms/cc. and remains at this level for a period of about 14 days.

Manometric measurements show that organisms from a peptone medium do not utilize sucrose. If washed and starved cells (from the peptone medium) are incubated with sucrose for 6 hours there is no increase in oxygen uptake in the presence of sucrose, and no reducing sugars are recovered. However, if cells from the sucrose medium are incubated with 60 mg. of sucrose, after an induction period of 80 minutes there is a gradual increase of oxygen uptake which is 74 per cent above the endogenous rate at the end of 300 minutes. At this time, there is recovered 11 mg. of reducing sugars.

The absence of added nitrogen in the sucrose medium suggests that *Colpidium* is capable of fixing atmospheric nitrogen. Preliminary manometric determinations indicate that, in fact, the organisms do fix atmospheric nitrogen.

REPORT ON LALOR FELLOWSHIP RESEARCH

A study of the hydrogenase systems of green and blue-green algae. ALBERT FRENKEL.

The capacity of various organisms to reduce carbon-dioxide and other substances by means of molecular hydrogen was investigated. It could be shown that the ability to carry out photoreduction of carbon dioxide with hydrogen is not restricted to several species of the Clorococcales among the algae, but can also be observed in at least one of the Volvocales and in two different genera of the Myxophyceae.

The organism belonging to the Volvocales which was studied was Clamydomonas Moewusii, Gerloff (isolated by Dr. L. Provasoli, and kindly supplied by Ralph Lewin). This alga pos-

sesses a very active hydrogenase and thus differs quantitatively from green algae like *Scenedesmus obliquus* which has been studied most extensively thus far. Clamydomonas grown aerobically can be adapted to carry out reduction of carbon-dioxide with hydrogen by incubating cell suspensions for thirty minutes in an atmosphere of hydrogen in the dark. In contrast to adapted suspensions of *Scenedesmus obliquus,* Chlamydomonas cells will tolerate up to 700 foot candles of unfiltered white light (Mazda bulbs) before reversion to photosynthesis will occur, whereas *Scenedesmus obliquus* will usually revert at about 100 foot candles of white light; once reversion occurs the cells have to be re-adapted again in the dark to reactivate the hydrogenase. At low partial pressures of oxygen (2–5 mm. of mercury), adapted suspensions of Chlamydomonas cells will carry out the oxyhydrogen reaction which on the basis of oxygen consumed follows the course of a first order reaction.

$$O_2 + 2H_2 = 2H_2O$$

A number of blue-green algae were also investigated as their capacity to carry out photoreduction. Two species were found which could be adapted successfully. One of these, a species of Chroococcus, has been isolated and has been grown in pure culture. It behaves very much like a unicellular species of Synechococcus studied previously. Adaptation of Chroococcus sp. to photoreduction can be accomplished after two hours of incubation in an atmosphere of hydrogen. The cells revert to photosynthesis at about 100 foot candles of white light in a similar manner as *Scenedesmus obliquus.* Their behavior differs, however, from Scenedesmus in that the blue-green alga can be re-adapted at low light intensities (20 foot candles) as long as the partial pressure of oxygen evolved by photosynthesis does not exceed approximately 1 mm. of mercury. Chroococcus sp. will also carry out the oxyhydrogen reaction in the dark.

These algae in addition to their capacity to reduce molecular oxygen in the dark and carbon dioxide in the light by means of molecular hydrogen are able to reduce other compounds with hydrogen. *Scenedesmus obliquus* will reduce quinone to hydroquinone in the dark as follows:

$$\text{Quinone} + H_{2(g)} = \text{Hydroquinone.}$$

At low concentrations of quinone (1.5×10^{-3} moles per liter), the reaction is of the first order and 95 to 100 per cent of the added quinone can be reduced in this manner.

Work is in progress to study the reduction of nitrate by means of molecular hydrogen in the dark and in the light, and to investigate intermediate compounds formed during the reduction of nitrate to ammonia.

Marine invertebrate phosphatases. C. Albert Kind.

Although many reports on the activity of vertebrate phosphatases have appeared in the literature, data on the phosphatase activity of marine invertebrate tissues is singularly lacking. Norris and Rama Rao (*J. Biol. Chem.* 108: 783 (1935)) demonstrated "alkaline" and "acid" phosphomonoesterase activity in echinoderms and mollusks and a relationship between "alkaline" phosphatase activity and shell formation in mollusks has been postulated by Manigault (*Ann. Inst. Oceanograph.* 18: 331 (1939)).

The present work was undertaken to provide data on the phosphatase activity of representatives of marine invertebrate phyla for which no such information is available. The tissues were homogenized with water saturated with chloroform (20 to 25 ml. solvent per gm. tissue) and allowed to stand at 5° for 20 hours. The mixtures were centrifuged and 1 ml. aliquots of the phosphatase-active supernatants were incubated at 28° for 24 hours with solutions of beta-glycerophosphate over pH range from 4.0 to 9.0. Contrary to a report by Norris these extracts are not inactivated by heating above 30° since samples incubated for 24 hours at 37.5° showed no loss in activity. Inorganic phosphate was determined by the method of Fiske and SubbaRow (*J. Biol. Chem.* 66: 375 (1925)).

All extracts studied showed optimum activity at p.H 4.5 to 5.0, and with the exception of extracts of Porifera and the one representative of Ctenophora, at pH 8.0 to 8.5. The activities are very low in comparison with those of vertebrate tissue and thus a relatively long incubation time is advisable. There appears to be a progressive increase in "alkaline" phosphatase activity with increasing biological complexity. Also of interest is an apparent increase in the *ratio* of "alkaline" to "acid" phosphatase activities expressed as mg.P per gm. tissue. The following values for this ratio are based on two representatives of each phylum: Porifera 0.075, Coelenterata 0.29,

C. tenophora 0.75, Echinodermata 0.94, Mollusca 1.6. Any phylogenetic significance in the increase of the "alkaline" to "acid" ratio must of course be based on a much larger number of observations but the possibility of such a relationship appears to be indicated.

Ribonucleic acid at cell surfaces and its possible significance. A. I. LANSING AND T. B. ROSENTHAL.

We have previously shown by means of Celite column chromatography and Ca^{45} labelling that a portion of mouse liver calcium is bound to a ribonucleoprotein. One of our students (Li) has shown that both calcium and ribonucleic acid are lost from the region of liver cell surfaces after in vivo administration of ribonuclease. The present experiments were designed to localize the peripheral ribonucleic acid in marine eggs.

Unfertilized and fertilized eggs of Arbacia, Asterias, Chaetopterus, and Echinarachnius were exposed to ribonuclease in sea water (controls to boiled ribonuclease), fixed in alcohol-formalin, imbedded in paraffin and sectioned at 4 micra, and stained for 2 to 5 minutes in 0.5 per cent toluidine blue.

The control unfertilized eggs showed a thin band of basophilia in both the cell cortex and in the vitelline membrane; this basophilia was more pronounced in the fertilized eggs. A moderate metachromasia was noted in the vitelline and fertilization membranes. The ribonuclease treated eggs showed a complete loss of basophilia in the cell cortex and in the vitelline membranes. The fertilization membranes retained a small amount of the blue dye while the red metachromasia was quite pronounced. We have concluded that the cell cortex of these eggs contains ribonucleic acid and that the vitelline and fertilization membranes contain ribonucleic acid with a second moiety responsible for the metachromasia. The latter, which may well be a polysaccharide sulfate ester, is under further investigation.

On the basis of Neuberg's impressive experiments (*Arch. Biochem.* 20: 185–210, 1949) which indicate that nucleic acids have a tremendous capacity for complexing with minerals, we have suggested that the ribonucleic acid at cell surfaces may be involved in the ion uptake mechanism of cells. This possibility is supported by a simple experiment on Elodea leaves.

The Elodea leaves were citrated to remove calcium, exposed to ribonuclease (controls to boiled ribonuclease) then to 0.02 M $CaCl_2$ or $SrCl_2$, plasmolyzed in 0.5 M sucrose and deplasmolyzed in pond water. This results in formation of numerous Ca and Sr oxalate crystals (cf. Mazia, *Biol. Bull.* 71: 306–323, 1936). However, the ribonuclease treated leaves showed almost complete inhibition of crystal formation, which may be due to failure of the calcium or strontium ions to be bound.

Determination of amino acids in invertebrates. JERRE L. NOLAND.

With the recent development of the microbiological procedures, it has become relatively easy to determine amino acids quantitatively in biological materials. To date, however, there are few reliable values for the amino acid composition of invertebrate tissues. In the present project the blood and muscle of the following animals are being analyzed for 18 amino acids:

Phylum	Species	Tissue analyzed
Annelida	*Phascolosoma gouldi* (Pourtales)	coelomic fluid
		muscle
Mollusca	*Mactra solidissima* Dillwyn	blood
		muscle
	Loligo pealei Lesueur	blood
		mantle
	Busycon canaliculatum Say	muscle
Arthropoda	*Limulus polyphemus* L.	blood
		blood clot
		muscle
	Callinectes sapidus Rathbun	blood
		muscle
Echinodermata	*Thyone briareus* (Lesueur)	muscle
Chordata	*Squalus vulgaris*	blood
		muscle

The blood samples were diluted with distilled water, and the protein precipitated according to the method of Hier and Bergeim (*Jour. Biol. Chem.* 161: 717 (1945)). The blood filtrate was adjusted to pH 6.8 and stored in the cold under toluene. The muscle tissue was isolated by dissection, washed in tap water, dried at 60° C. in vacuo and powdered. Aliquots of the powder were hydrolyzed with 4 N HCl or NaOH, adjusted to pH 6.8, diluted to volume and stored under toluene in the cold.

The determinations were made by a microbiological procedure, using *Leuconostoc mesenteroides* P-60 or *Leuconostoc citrovorum* 8081 grown on medium VI of Steele et al. (*Jour. Biol. Chem.* 177: 533 (1949)). The lactic acid produced after 72 hours incubation was titrated electrometrically with 0.02 N NaOH.

Tentative values have been obtained for the amino acids tryptophan, tyrosine, histidine, leucine and proline. From these data it appears that the blood of Callinectes has the highest concentration of "free" amino acids, followed in order by Loligo, Phascolosoma and Squalus. The blood filtrates of Mactra and Limulus appeared to be practically free of these amino acids. The results of the muscle analyses indicate that invertebrate muscle must be similar in composition to muscle from vertebrates. This is in accord with the scattered data already published concerning the amino acid composition of muscle.

The release of radioactive Ca^{45} from muscle during stimulation. ARTHUR A. WOODWARD, JR.

The release of Ca^{45} from frog muscle was studied. After all attempts to get a usable amount of Ca^{45} into isolated single muscle fibers had failed, experiments were thereafter performed with whole sartorius muscles. These were carefully dissect to avoid the slightest injury and then immersed for 3 to 4 hours at 5° C. in Ringer solution containing 5 microcuries per ml. of Ca^{45}. The muscles were then removed, rinsed, and washed in successive equal portions of fresh Ringer solution; the time spent in each washing bath was the same in any given experiment, usually either 1 min. or 3 min. After 5 to 7 of such washings the muscles were then stimulated electrically while immersed for the same length of time in a final bath of Ringer solution. Each of these washings was then dried and the amount of radioactive material present determined with a Geiger-Muller counter.

The amount of Ca^{45} given off to the washing baths dropped rapidly in successive samples to a low level and then continued to decrease slowly and rather uniformly. Electrical stimulation caused increases of from 30 per cent to 200 per cent in the amount of Ca^{45} released to the bathing medium. Analysis of curves obtained from plotting the data leave little doubt that the Ca^{45} thus released came from the muscle fibers rather than from the interfibrillar spaces. This was confirmed by experiments in which the first period of stimulation was followed by alternate washings at rest and during stimulation. Stimulation always produced an increase in Ca^{45} released, and that given off during rest always dropped back to its previous level.

In other experiments carefully matched muscles were selected and exposed to increasing amounts of stimulation by increasing the frequency of stimulation over a given time interval. Increase in the amount of stimulation always produced an increase in amount of Ca^{45} released to the bathing medium. It is concluded that electrical stimulation of frog muscle causes a release of free Ca ions from the protoplasm of the muscle fibers.

Proteolytic enzymes in cytoplasmic granule preparations of Arbacia eggs. ARTHUR A. WOODWARD, JR.

Preliminary but very scant observations show the presence of proteolytic enzyme activity in preparations of cytoplasmic granules obtained from unfertilized Arbacia eggs by fractional centrifugation (partial fractionation only) after the method of Harris (1942). Only one preparation of granules suitable for satisfactory enzyme assay was obtained. This sample contained all the granular fractions of the cytoplasm, but was free of whole cells, nuclei, membranes, and other extraneous materials. The preparation had a low but significant protease activity which was increased from 35 per cent to 50 per cent upon the addition of cyanide. The addition of Ca ions to the preparation had no detectable effect on the enzyme activity. The sample was too small to admit of further fractionation of the granules with profit. The method of Anson (1938) was used for assay of proteolytic enzymes.

Electrophysiological measurements on the eyes of Limulus and Loligo. V. J. Wulff.

1. Measurement of asymmetry and action potentials.

All attempts to obtain a measurement of asymmetry potentials in the lateral eye of Limulus met with negative results. It was concluded that the orientation of an asymmetry potential, if it exists at all, must have been normal to the axis of the electrodes. This interpretation is indicated by the morphology of the sense cells and merits further investigation.

Similar measurements on the excised eye of the squid, *Loligo pealei,* yielded asymmetry potentials ranging from 5 millivolts at the beginning of an experiment to ca. zero mv. at the end of an experiment. The asymmetry potential often reversed its polarity, so that an initially negative corneal electrode would become positive, with a subsequent decline to zero. Retinal action potentials, obtained in response to constant intensity, constant duration light stimuli at fifteen minute intervals during the course of the experiment, indicated that the magnitude of the voltage wave varied with the magnitude of the asymmetry potential. Reversal of the asymmetry potential was accompanied by reversal of polarity of the action potential. The lens and outer covering of the eye ball contributed about 50 microvolts to the overall potential. Both asymmetry and action potentials decline rapidly to zero under conditions of partial or total oxygen lack.

2. Measurement of retinal action potentials and optic nerve action potentials.

Measurements on the lateral eye of Limulus are abstracted in detail elsewhere. Similar measurements on the median eye of Limulus indicated parallelism with the lateral eye. The median eye was excised completely with its nerve and introduced into an oil-filled capillary tube. The response of the sense cells of this preparation duplicated the results obtained from the lateral eye.

Similar measurements on the excised squid eye yielded negative results with respect to measurement of optic nerve activity. It was concluded that the optic nerve fibers become rapidly inactive upon removal of the eye from the organism or that the magnitude of the nerve action potentials is so small as to be undetectable with the equipment used.

3. The effect of some chemical agents on the responses of the lateral optic pathway of Limulus.

The addition of procain hydrochloride to the fluid in the rear compartment of the eye chamber (final concentration — 0.02 per cent) immediately abolished nerve activity and slowly caused a decline in phase two of the retinal response.

The addition of sea water containing excess KCl (ten times that present in normal sea water) abolished nerve activity and caused a slow decline of the retinal response, affecting first the second phase and subsequently affecting the first phase. Before the nerve activity ceased it was observed that the "silent period" in the nerve discharge following an initial rapid burst of impulses, was greatly reduced or absent. The effect of KCl was reversible.

The addition of veratrine hydrochloride (final conc. 1 part per 500,000, 1,000,000 and 2,000,000) abolished the response of the optic nerve and produced an enhancement of the retinal action potential, affecting both phases, particularly the declining limb of the second phase.

4. The effect of light adaptation of the responses of the lateral optic pathway of Limulus.

Progressively increasing light adaptation produces a reduction followed by an increase in latency of both retinal and nerve response and a gradual encroachment of the second phase upon the first phase of the retinal response.

Characteristics of electrical activity in the lateral optic pathway of Limulus. Verner J. Wulff.

Illumination of the lateral eye of *Limulus polyphemus* elicits a potential change, the retinal action potential. The magnitude of the retinal action potential varies with the logarithm of the intensity. The retinal action potential is followed by the appearance of activity in the optic nerve. Both retinal and optic nerve responses exhibit latencies. The latency of the latter exceeds that of the former by an interval which varies inversely with the intensity and with the magnitude of the retinal response. This difference in the latencies of the retinal and optic nerve responses is called the retinal-nerve interval. In many experiments the R-N interval progres-

sively increases as the intensity of illumination decreases; in others the R-N interval increases with decreasing intensity up to a point and then decreases upon stimulation with still lower intensities. This peculiar relation has a parallel in the retinal response. The time for the retinal response to reach its maximum, called peak-time, exhibits a similar relation to intensity. Further study revealed that the retinal response is dual in nature. A single smooth response is evident upon stimulation with low intensities. Stimulation with higher intensities elicits a second wave of potential superimposed on the first, which grows rapidly in magnitude and eventually obscures the first phase. It is suggested that this duality of the retinal response may possibly indicate two types of sense elements (for which there is no supporting evidence) or that the sense cells exhibit a dual response, analogous to peripheral nerve. The increase of the R-N interval with lowered intensity of stimulation and, consequently, with lower magnitude of the retinal response, lends support to the hypothesis that local action currents caused by the retinal potential initiate activity in the nervous elements of the optic pathway.

PAPERS PRESENTED AT THE MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

June 24, 1949

Specificity of desoxyribonucleases and their cytochemical application.[1] Jay Barton II and Daniel Mazia.

The specificities of Desoxyribonucleases (DNases) are of special interest because of (1) the insight they may give us into the structure of DNA, and (2) their possible application to cytochemical study of DNA.

The initial observation suggesting that there may be more than one qualitatively distinct DNase was made while developing a new method for the estimation of the enzyme. This new method is based on the precipitation of high polymer nucleic acid by protamine and the spectrophotometric determination of the remaining low polymer. No good correlation could be found between the activity as measured by this method and when measured by the usual viscometric methods, even though a crystalline preparation was used. Several points of difference were found. (1) The pH optimum when measured by the method of Barton and Mazia is about 5.6; when measured by the viscosity method the optimum is about 6.9. (2) The reaction rates are of an entirely different character. The protaminc method shows a reaction of the zero order, while with the viscosity method the reaction curve approaches that of a first order reaction. (3) With the same crystalline preparation (Kunitz method, prepared by Worthington Biochemical Lab.) the limiting enzyme concentration when measured by the viscosity method is reached at about 0.1 microgram per ml. This concentration is just at the lower limit of detectability by our new method. The activity measured by this method is still proportional to enzyme concentration about 10 microgram per ml. (4) When the activity of tissue homogenates is determined by both of the methods, the ratio between "viscosity" activity and "protamine" activity is found to vary. This last observation suggests that two enzymes are involved rather than multiple specificities of a single enzyme.

Kunitz's method concentrates the enzyme measured by viscosity-lowering, but two crystalline preparations that have been tested both contain a small contamination of the activity measured by the protamine method. In every experiment made thus far, the viscosity lowering is complete before protamine-soluble DNA appears.

It is suggested that the DNase measured by the protamine method is specific for the linkages primarily responsible for polymerization of nucleotides while the viscosity-lowering enzyme is specific for another class or classes of linkages responsible for structural viscosity. From this would follow a picture of the DNA macromolecules essentially similar to that of Gulland.

Initial experiments applying the two specificities to cytochemical analysis of the mode of attachment of DNA in the salivary chromosomes of Chironomus have been made. Loss of DNA from the chromosome can be attributed to the same activity as that measured by the protamine method. The conclusion that DNA may be removed from the chromosome by rupture of internucleotide linkages is understandable only in the light of what is known of the altered solubility of low-polymer DNA-protein complexes. Both the DNA and the histone are removed together, leaving a "residual" intact chromosome behind. The inference is that the attachment to the chromosome is weak enough to be broken when the size of the DNA-basic protein complex is changed.

Physiology of tracheal filling in Sciara larvae. M. L. Keister and J. B. Buck.

During each larval instar of Sciara, the air-filled tracheae become enclosed in larger, liquid-filled, coaxial tubes which are to form the tracheal system of the next instar. At moulting the old gas-filled tracheae are withdrawn and shed with the body cuticle. Three to eight minutes later, the liquid column breaks at some single point within one of the main trunks, and gas

[1] Work supported by a grant from the American Cancer Society, recommended by the NRC Committee on Growth.

rapidly fills the entire new system. The gas does not enter by way of the spiracles. The liquid is withdrawn into the blood and tissues. Filling of the tracheae with gas will occur in a normal manner in larvae submerged in oil or water in the absence of visible gas. Gas-filling is delayed by exposure to nitrogen, carbon dioxide or carbon monoxide containing less than 0.5 per cent of oxygen, and indefinitely inhibited by completely oxygen-free gases. High concentrations of carbon monoxide in the dark do not inhibit filling. Filling is inhibited during and for some time after exposure to low temperature. Diffusion, release of hydrostatic pressure, increase in tissue osmotic pressure, and muscular activity are shown to be inadequate to explain tracheal filling. It is suggested that an aerobic metabolic process is involved.

Bioelectrical models of energy transformation in nerve. T. C. BARNES AND R. BEUTNER.

A modification of the elongated oil-cell model of bioelectrical potential previously described to the Society of General Physiology (*Biol. Bull.* **95**: 281, 1949) has openings along the tube to permit recording of the wave produced by acetylcholine at various distances away from the point of application and at various inter-electrode distances. The wave behaves like a nerve impulse between two nodes of Ranvier (Stampfli, XVII Internat. Physiol. Cong., p. 218, 1947). The recent rediscovery of nodes in fibres of the brain (Allison, *Nature* **163**: 449, 1949) suggests that our tube model applies to the electroencephalogram. The synapse is represented by a low-resistance oil in a U-tube at the end of the long tube. This U-tube synapse responds readily to acetylcholine in contrast to the axon where resin is added to duplicate insensitivity to chemical mediators. Sensitization to histamine is produced in a stationary model by adding lauryl sulfonate to triacetin on which 0.05 per cent histamine give 20 mv. negative but has no effect in absence of lauryl sulfonate. The lauryl sulfonate solution clears on addition of acetylcholine or histamine suggesting colloidal change. Mixtures of guaiacol and resin kept for several weeks gradually lose their reaction with acetylcholine (model of aging). The role of the oils is largely physical since bromobenzene gives the same effects as nitrobenzene. All nerve substances studied give disappointing results except alkaloids (acetylcholine). 0.05 per cent ATP generates only 10 mv. positive on guaiacol in contrast to 35 mv. negative produced by acetylcholine. The positivity of ATP fails to sensitize oils further to the chemical mediator.

The effect of ultraviolet on green algae and isolated chloroplasts. A. S. HOLT AND W. A. ARNOLD.

A comparative study of the effects of ultraviolet (2537 Å) on various measurable processes occurring in algae and in chloroplast preparations shows wide variations in sensitivities. With four day old cultures of *Chlorella pyrenoidosa* and *Scenedesmus* D_1, it was found that the photosynthetic mechanism of Chlorella is far more sensitive than that of Scenedesmus. Endogenous respiration in both algae is comparatively untouched as compared to photosynthesis, while the oxidation of glucose added to starved Chlorella cells is many times more sensitive than photosynthesis. In Scenedesmus the same dosage of ultraviolet light caused the same percentage inhibition of photosynthesis, of photoreduction, and of oxygen evolution from p-benzoquinone solutions. Oxygen evolution by isolated chloroplasts and chloroplast fragments is also inactivated as shown by manometric measurements with ferricyanide as the oxidant, and by dye reduction measurements with 2,6 dichlorbenzenone-3-chlorophenol. The catalase activity of chloroplasts is not affected by doses that completely inactivate oxygen evolution. Comparative studies of the effects of the poisons HCN and NH_2OH on irradiated and untreated Scenedesmus show that both poisons reduce the rate of photosynthesis of the irradiated cells to the same percentage as they do the normal cells. Survival as measured by colony formation following irradiation is far more sensitive than photosynthesis.

Chemical induction of bud formation in plant tissues. FOLKE SKOOG AND CHENG TSUI.

Results from experiments on the interaction of indoleacetic acid and phosphate in growth and organ formation in tobacco tissue cultures led to tests on the effect of adenine and its derivatives on bud formation.

It was found that adenine supplied to the nutrient medium causes bud formation in tobacco callus and stem segment cultures under conditions where controls formed none, and increased the number of buds where the controls formed a few. The number increases with concentration of adenine up to 40 mg. per l. and may reach 40 buds on a 150 mg. piece of tissue. IAA prevents bud formation, it stimulates callus growth, and induces root formation. Mixtures of the two compounds in high Ad per IAA ratios may permit formation of both buds and roots. Rel. high concentrations of both compounds cause rapid growth of the tissues without organ formation. Many other purines have a similar but less effect than adenine on bud formation.

A controlling influence of the adenine-IAA ratio on growth and organ formation has also been established in tissues from horse radish and carrot. However, the concentration ranges for activities varies with the species.

A brief summary of tissue contents and distribution of phosphates and auxin in relation to the treatments and morphological changes which they produce will be presented.

Effects of ultra-rapid freezing on mammalian erythrocytes. B. J. LUYET.

Smears of oxalated ox blood, in layers about 60 micra thick, cooled at a rate of some hundred degrees per second by immersion in liquid nitrogen, and rewarmed at a velocity of the same order by immersion in physiological saline at room temperature, showed only a slight hemolysis and furnished, upon centrifugation, a red-cell content of about 72 per cent that of normal untreated blood. When similar smears were exposed for some 20 seconds, either during cooling or during rewarming, to the crystallization temperatures (a few tens degrees below zero), hemolysis was heavy and the hematocrit readings indicated only some 4 per cent non-hemolyzed red cells. Varying the time in liquid nitrogen from 10 seconds to 2 hours did not change the percentage of surviving cells. The possibility of an exosmosis of water, during freezing through the relatively large surface areas of such small cells may account for the fact that some erythrocytes escape injury when freezing is relatively slow, and the absence of crystalline ice in all probability explains the preservation of most cells after ultra-rapid freezing; but whether the destruction of more than a fourth of the cells upon ultra-rapid freezing is caused by the incomplete prevention of ice crystals remains to be ascertained. (If equipment and material are available, a demonstration will be made of the "vitrification" of aqueous solutions, of the prevention of hemolysis in ultra-rapid freezing and of the methods used, in general, for obtaining, controlling and recording cooling or rewarming velocities up to 20.000 degrees C. per second.)

Photosynthesis and photoreduction by a species of blue-green algae. ALBERT FRENKEL, HANS GAFFRON, AND EDWIN H. BATTLEY.

A naturally occurring enrichment culture of Synechococcus sp. found on Angelica Point in Buzzard's Bay was investigated for its capacity to carry out photosynthesis and photoreduction. Under aerobic conditions the cells carried out normal photosynthesis with a photosynthetic quotient of $\Delta O_2/\Delta CO_2$ of $1.08 \pm .02$.

Under anaerobic conditions in the presence of hydrogen the cells can be adapted to carry out photoreduction of carbon-dioxide by means of molecular hydrogen. In general these blue-green algae behave in the same way as Scenedesmus strains in which this process was first observed among algae. Oxygen and high light intensities will reverse the adaptation reaction so that the cells will again carry out normal photosynthesis. Sodium sulfide (10^{-3} M per L.) will inhibit the adaptation reaction in the presence of hydrogen, however, when sodium sulfide is added after adaptation has taken place it will stabilize photoreduction against reversal by high light intensities. Preliminary experiments indicate that added sulfide will not disappear as long as photoreduction is carried out in the presence of molecular hydrogen. However, when hydrogen is replaced by nitrogen and sodium sulfide is added, the latter will disappear in the light with the simultaneous reduction of carbon-dioxide. For each molecule of carbon-dioxide two molecules of sulfide will be oxidized. As soon as all the sulfide is oxidized the cells will revert to normal photosynthesis. Cultures of Scenedesmus sp. adapted to photoreduction are also able to reduce carbon-dioxide with the simultaneous oxidation of sulfide in the light, in the same way as Synechococcus sp. This process may be similar to that occurring in some of the sulfur bacteria.

Riboflavin-sensitized photo-oxidations and their significance in plant physiology. ARTHUR W. GALSTON.

In the course of investigations to determine the mechanism of the light inhibition of growth of plant stems, it was discovered that riboflavin, when applied to plants in physiological concentrations, exerts a profound inhibitory effect on growth. This inhibition is manifested only in the light, and was subsequently found to be due to a riboflavin-sensitized photooxidation of the plant growth hormone, indoleacetic acid (IAA). *In vitro,* this reaction is first order with respect to disappearance of IAA. It requires oxygen, one mol of O_2 being absorbed and 1 mol of CO_2 being released per mol of IAA photoinactivated. The oxidation product is a physiologically inactive, brown-red material, with a melting point of about 150° C., and an approximate empirical formula of $C_{12}H_{10}NO$. Its structure is still under investigation.

In addition to IAA, the following compounds may undergo riboflavin sensitized photo-oxidations: all indole-containing compounds, histidine, methionine, various enzymes (including urease, tyrosinase and α-amylase), immune proteins and bacteriophage T6r. Lumichrome is less effective as a sensitizer than is riboflavin, and lumiflavin is not at all effective. The activity of d-amino acid oxidase of hog kidney, which contains a riboflavin-adenine dinucleotide prosthetic group is, however, unaffected by light.

Avena coleoptile tips contain about 30 micrograms of riboflavin per gram dry weight. If the diffusible auxin of *Avena* coleoptiles is gathered into agar blocks containing traces of riboflavin, this auxin is rapidly photoinactivated. It therefore seems probable that, *in vivo,* riboflavin may sensitize the photoinactivation of IAA. If this is true, then riboflavin may be involved in the phototropic response of *Avena* coleoptiles. Although several of the published action spectra for phototropism contain a double maximum suggestive of a carotinoid receptor, they do not rule out the participation of riboflavin, which has its visible absorption maximum at about 450 mμ, in the region of highest phototropic effectiveness.

It is further suggested that this type of reaction may explain the often reported fact that tissue exposed to strong light has a lower auxin content than does unilluminated tissue. The implications of such reactions for plant growth are numerous.

Studies on the rigor resulting from the thawing of frozen skeletal muscle. S. V. PERRY.

When frozen frog sartorious muscle is allowed to thaw at room temperature it goes into a rigor which is characterized by spontaneous shortening resulting in a decrease in length of 70 per cent after 10 to 15 minutes and an obvious synaeresis producing a loss of weight of 35 per cent, changes which bear considerable resemblance to those occurring during the contraction of actomyosin threads. Although a load of 8 gm. is sufficient to prevent the shortening, when the load is removed the muscle will shorten to the normal extent even after it has been kept thawed and loaded for as long as 20 minutes.

The shortening takes place if muscle is frozen in the resting condition, after exhaustion by direct electrical stimulation, during isometric tetanus, and after it has been treated with inhibitors such as azide, cyanide, copper, hydrogen peroxide, p-chloromercuribenzoate, iodoacetate, and 2:4 dinitrophenol. It can be prevented, if before freezing, the iodoacetate poisoned muscle is exhausted, or left fixed at its resting length for several hours in N_2. After such treatment synaeresis is also absent on thawing. Shortening can be produced in these muscles by immersing them in a solution containing 0.0029 M ATP, 0.05 M KCl and 0.0005 M $MgCl_2$. Prolonged exposure to p-chloromercuribenzoate renders muscle inexcitable but the ATPase activity of myosin prepared from it and the shortening of thaw rigor are little affected. Similar although less clearcut results were obtained with hydrogen peroxide, indicating that in view of the effectiveness of these inhibitors on the enzyme activity of myosin *in vitro,* their action on the muscle cell must be confined to the membrane, or the myosin of the fibrils must be in some way protected from reacting with the inhibitors.

The phenomenon seems to be an in situ example of the synaeresis of actomyosin, but it does not necessarily follow that such system is responsible for muscle contraction. In fact there is a suggestion from this study that in muscle frozen while it is tetanised into maximal shortening,

the actomyosin is still largely unsynaeresed, because synaeresis obviously takes place on thawing with a loss in weight only 27 per cent less than that shown by resting muscle.

KENNETH E. FISHER.

Considerable interest attaches to investigations dealing with the net movements of organisms to or away from some source of stimulation and to the mechanism of the aggregations of organisms in a particular region of a non-uniform environment. As an aid in such investigations, we have considered methods of describing the movements of organisms which would permit the construction of the hypothetical path traced out by an organism in a simplified situation. A rigid quantitative procedure has been devised by which this can be done. Using it the conditions required to predict the uniform distribution of organisms throughout a uniform environment have been determined as well as the nature of the "biases" necessary to bring about an aggregation of organisms in a non-uniform environment.

Time course studies of photosynthesis and respiration in unicellular algae using the platinum electrode with time selection. F. S. BRACKETT AND R. A. OLSON.

Observations of oxygen tension are obtained during respiration and photosynthesis in unicellular algae by means of a new method of oxygen determination employing an alternating potential and an accurate selection of time with the static platinum electrode. This method [1] provides accurate and reproducible determinations at 10-second intervals without the need of stirring or of moving components. By using small tubular cuvettes (2 mm. diam.) suspensions can be radiated from opposite sides by means of a dual monochromator system such that the intensity gradient due to absorption is minimal and all cells receive nearly equal radiation. This avoids the intermittent radiation of cells at widely varying intensities peculiar to the total absorption method in conventional manometry where unilateral radiation and stirring are employed. Typical time course records using this new method are presented and evaluated.

[1] Olson, Brackett, and Crickard, *Jour. of Gen. Phys.* (in press).

COMPARATIVE SEROLOGY OF SOME BRACHYURAN CRUSTACEA AND STUDIES IN HEMOCYANIN CORRESPONDENCE [1]

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Introduction

Researches in systematics may be conducted by comparing the serum or corresponding body proteins of organisms using serological methods. The underlying principle of serological systematics is that the proteins compared are representative of the organisms producing them in the same sense that their corresponding structures are and for the same general reasons. Of the serological reactions used for such researches the precipitin reaction has undoubted advantages. An extension of the previous precipitin studies in the serological systematics of Crustacea and related problems is the content of this report.

General Materials and Methods

Antigens

The sera of the Crustacea compared in this study were in part provided by Dr. Alan A. Boyden [1] who gathered them during summers over a period of years at various biological stations along the Eastern coast of the United States and elsewhere, namely, Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, 1936; U. S. Bureau of Fisheries Laboratory, Beaufort, North Carolina, 1936; Tortugas Laboratory, Carnegie Institution, Washington; Key West, Florida, 1932, 1934, 1936, 1939; also at the Marine Laboratory, Citadel Hill, Plymouth, England, 1939, 1948.

In addition to the samples of sera, the author had access to Dr. Boyden's original records made at the time of collection, plus the correpondence incidental to the collection and identification of particular samples. These records were of great help in orientation to the work and in providing useful hints and suggestions which saved countless hours when the author did his own field collecting.

Useful serum samples were secured by the writer in the field at Barnegat Bay and Delaware Bay, New Jersey. Samples of European crustacean sera were collected in the Summer of 1948 by the author at the following European biological laboratories: Museum National D'Histoire Naturelle Laboratoire Maritime, Dinard, France; Universite de Paris, Biologie Marine, Laboratoire Arago, Banyuls-sur-Mer, France; and the Stazione Zoologica, Napoli, Italia.

[1] This investigation was supported in part by a grant from the Research Council of Rutgers University. The author wishes also to express his sincerest thanks to Professor Alan A. Boyden, Director of the Serological Museum of Rutgers University, for his advice and encouragement, and for providing many of the serum samples used in this study.

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In general the blood was taken from the crabs by removing the 5th pereiopod and permitting the blood of the organisms to drain into porcelain pans or glass crystallizing dishes. The crabs were held over the collecting pans by means of an ordinary clamp attached to a cross bar supported between two vertical stands. In the case of the long-tailed Crustacea such as crayfish and lobsters, the blood was collected by slitting the abdomen ventrally where it joins the thorax and holding the organism by hand over a large collecting funnel making sure the posterior end of the abdomen was pressed firmly to the lip of the funnel as an insurance against sudden abdominal flexures. It is necessary to occasionally break the jelly-like clots that form at the openings from which the blood is draining in order to obtain good yields from each animal. In those cases where the organisms were small the blood was taken from convenient points within the body cavity by means of needle and syringe. Piercing the body at the base of the appendages usually gave good yields. It is interesting to note here that placing the needle in the heart or in the pericardium gave poorer results than when a sinus some distance from the heart was tapped.

TABLE I

List of the species of Crustacea used as antigens

Acanthocarpus alexandri Stimpson
Callinectes marginatus (Milne-Edwards)
Callinectes sapidus Rathbun
Cancer anthonyi Rathbun
Cancer borealis Stimpson
Cancer irroratus Say
Cancer magister Dana
Cancer pagurus L.
Carcinus maenas L.
Geryon quinquedens Smith
Maia squinado Rondelet (Herbst)
Menippe mercenaria (Say)
Mithrax verrucosus Milne-Edwards
Ocypode albicans Bosc.
Panopeus herbstii Milne-Edwards
Panulirus argus (Latreille)

The collected bloods were permitted to clot and the sera to be expressed. Centrifugation at 2000–3000 r.p.m. for 20 minutes was usually employed to clear the sera. It is feasible to hasten the expression of serum by wrapping the clot in a double thickness of clean, fine-mesh, bolting or cheese cloth and twisting the wrapping by hand. The sera were sterilized by Seitz filtration, bottled in serum vials and stored at $3° \pm 1°$ C.

Table I is a list of the Crustacea whose sera were used as antigens in this work. The identifications of North American Crustacea were made with the works of Pratt (1936), Faxon (1898), and the remarkable publications of Rathbun (1917, 1925, 1930, 1937). The French fauna was checked against Bouvier (1940). The fauna of other European countries was identified in the works of Borradaile (1907), Pesta (1918), and Thiele (1935).

Antisera

All antisera were produced in rabbits. Intravenous and/or subcutaneous routes of injection were employed in the production of antisera. Inasmuch as it is impossible to ascertain at present the degree to which a rabbit will respond to a given amount of antigen, and since this response is perhaps the greatest variable in present day serological studies, what was believed to be minimal amounts to produce a good response were used. Generally 0.25 cc. was given on the first injection followed on alternate days with three 0.5 cc. injections. In every instance good, usable antisera were obtained when the hemocyanins were injected in these doses. In some cases a presensitization technique was employed to improve the strength (i.e. precipitating capacity) of the antisera. These rabbits were given 0.25 cc. of antigen intravenously and then permitted to rest for 30 days. This was followed by a series of 4 subcutaneous injections given on alternate days.

TABLE II

List of antisera

Antiserum	Homologous antigen	Remarks
I–63	*Menippe mercenaria* 36-A	Prepared by Dr. Alan A. Boyden Rutgers University
I–76	*Panulirus argus* 39–1	
I–77	*Panulirus argus* 39–1	
I–78	*Cancer borealis* 3a1	
I–86	*Callinectes sapidus* 47–1	
I–87	*Callinectes sapidus* 36–1	
I–94	*Cancer borealis* 1a	
I–95	*Maia squinado* No. 5	
I–97	*Cancer pagurus* No. 3	
I–98	*Menippe mercenaria* 36–A	
I–99	*Ocypode albicans*	
I–100	*Geryon quinquedens* 36–1	
I–101	*Cancer irroratus* 36–1	
I–105	*Geryon quinquedens* 36–1	
I–106	*Cancer irroratus* 36–1	
I–107	*Acanthocarpus alexandri*	
I–110	*Geryon quinquedens* 39–1	
I–114	*Callinectes sapidus* 47–1	

Table II is a list of antisera prepared and utilized by the author except where specially annotated.

Bleeding the rabbits to secure the antisera was accomplished in either one of two ways. Small samples of blood were withdrawn from the central artery of the ear, by using a number 22 gauge needle and syringe. For complete bleedings blood was withdrawn directly from the heart by cardiac puncture. Size number 18 gauge needles and 50 cc. syringes were used in this latter procedure. All antisera were centrifuged, sterile filtered through Seitz filters, and stored in the refrigerator until used.

Method of testing

The Libby photronreflectometer (1938) was utilized exclusively in the measurement of turbidities developed as a result of the interaction of antigens and antibodies. The technique employed was essentially the same as that described by Boyden and DeFalco (1943). Minor variations in technique as developed by the writer were matters of convenience and did not represent any major changes in their method of making the dilution series (see Figs. 1 and 2) of the antigens, nor in the use of the machine. Two recent papers (Boyden et al., 1947; Bolton et al., 1948) have analyses of the performance of the photronreflectometer and report the conclusion that for white precipitate systems, which include all the precipitin reactions, the instrument is unsurpassed at present in its sensitivity and range of usefulness in studying the characteristics of precipitates.

For all tests the procedure in which the amount of antiserum is held constant and the amount of antigen is varied was employed. The reacting cells of the photronreflectometer have a 2 cc. operating level and this volume was used in all testing. Final volume for each antigen dilution was always 1.7 cc. to which 0.3 cc. of immune serum was added to make up the 2 cc. volume Turbidities (i.e. galvanometer readings) inherent in the fluid of the antigen dilution, and those due to dirt or blemishes on the glass of the reacting cells plus the turbidity characteristic for 0.3 cc. of each antiserum used were deducted from the total turbidity developed in each reacting cell. For these reasons the resultant turbidities can be considered as those due to the interaction of antigen and antibody.

The range of antigen dilutions regularly employed was between 4000 and 1 gamma of protein per cubic centimeter or solution, or in terms of dilutions of protein from 1:250 in doubling series to 1:1,024,000. When necessary the range of antigen dilution was extended. For the sake of convenience in plotting the results of the reaction on graphs, the antigen dilution cells were assigned numbers in a chronological sequence with cell No. 1 containing the greatest concentration of antigen. Figure 1 shows graphically a typical dilution sequence.

For all of the work in this paper the precipitin turbidities were those developed during 20 minutes incubation in a dry-air incubator maintained at 38° C.

The titration curve

The graph of the turbidities developed for serological tests is usually made in this laboratory with the turbidities plotted as the ordinate and the antigen dilution as the abscissa with the greatest concentration of antigen nearest the x-y axes intersect. The turbidities rise to a maximum, then decline again to a minimum following generally a normal distribution curve. By assigning unit distances between the geometrically changing antigen concentration values along the abscissa, the curves as plotted are more or less symmetrical. Skewness toward the region of antigen excess is quite common. Variations in the amount of kurtosis have also been observed. The amounts and kinds of both antigens and antibodies in the solutions combine to provide many variations of this type of frequency distribution. Figure 2 represents an idealized titration curve. The numerical value used to characterize any one particular curve for comparative purposes is that obtained by summating the turbidities over the whole reaction range. This value is proportional

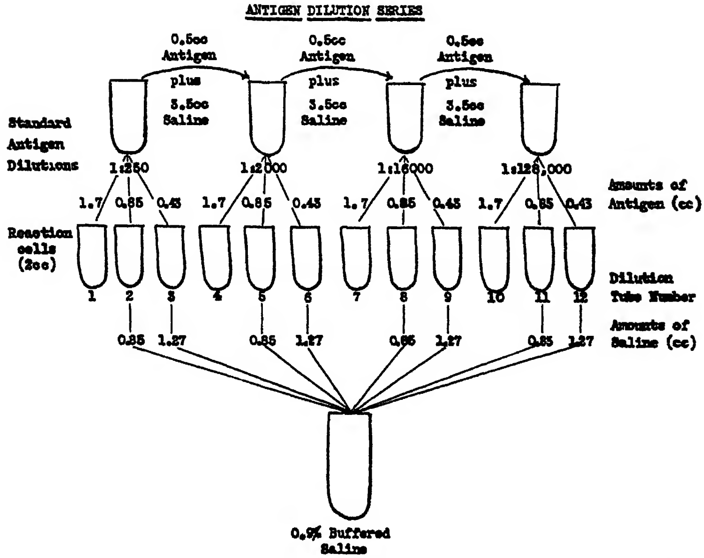

FIGURE. 1. A diagram of the technique used in preparing a typical doubling-dilution series of a given antigen for use in the Libby Photronreflectometer. The initial standard antigen dilution (1:250) is prepared directly from a serum of known protein concentration. The subsequent standard antigen dilution tubes are prepared from the first. Each reaction cell has a constant antigen dilution volume of 1.7 cc. to which is added and mixed a constant volume of 0.30 cc. of antiserum (a procedure).

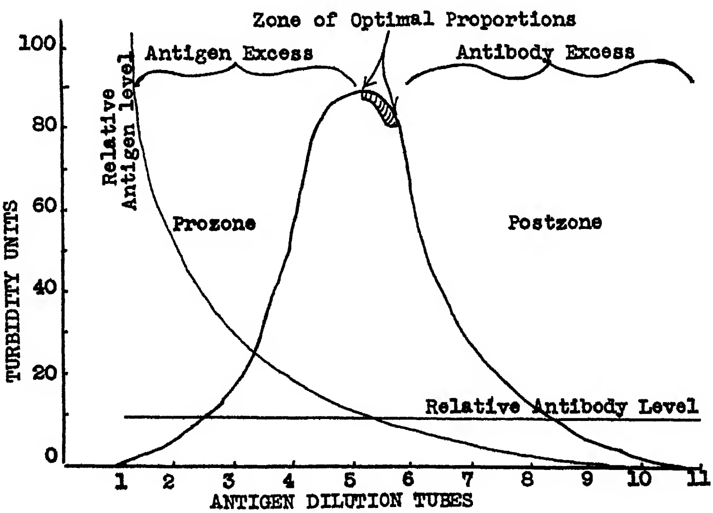

FIGURE 2. A diagrammatic representation of a typical titration curve obtained by using the Libby Photronreflectometer as the turbidimeter, and the technique of reacting varying antigen dilutions with a constant amount of antibody. Included also are appropriate curves to portray the relative antigen and antibody levels in each antigen dilution tube. For these latter curves the turbidity units of the ordinate axis do not apply.

to the area under the curve and provides an easily obtained statistical index of a very complicated biochemical system.

For all titrations the dilution medium was 0.9 per cent NaCl buffered with M/15 phosphate salts (Sorensen's solution) (Evans, 1922) such that the buffer was in a final concentration of M/150. The pH range for the tests was between 7.05 and 7.15.

Antigen Correspondence

The continuous analysis of the properties of both of the primary reagents used in serological work is necessary if the investigations are to be considered critical. This problem is especially pertinent to workers in the field of serological systematics since they must be certain that the sera or proteins or organisms used in their tests are unchanged from the native state, or if changes have occurred because of prolonged storage or other physical or chemical factors, they must be prepared to correct for it.

The sera of Crustacea contain one principal protein, hemocyanin (Allison and Cole, 1940). They are excellent antigens when injected into the rabbit. They are usually considered to be relatively pure systems. However, electrophoretic patterns (Cohn and Edsall, 1943), sedimentation constants (Dawson and Mallette, 1945; Redfield, 1934), and $(NH4)_2SO_4$ precipitation (Bolton, personal communication) all indicate that this single serum protein may be composed of several molecular "species" of hemocyanin. The hemocyanin molecules are large with molecular weight ranging from 300,000 upward to several millions as calculated by Svedberg and his collaborators from data obtained by using the ultracentrifuge methods of sedimentation velocity and sedimentation equilibrium. A glance at almost any titration curve of the hemocyanin antigen-antibody system (Fig. 3) will show irregularities and disturbances in the modality of the plot. Occasionally a titration will show two distinct modes. These variations from a single mode frequency curve have long been considered as evidence of the probable presence of more than a single kind of antigen or antibody in the system. It is quite likely that the above mentioned molecular "species" are responsible for the stimulation of more than a single principal kind of antibody in the rabbit serum. This, of course, does not preclude the possibility that a single molecular "species" could stimulate the production of two or more distinct kinds of antibodies. Until such time as this can be proven, it is simpler to assume that a single kind of antibody is produced against each kind of antigen, and that the appearance of additional modes in the titration curve of any antiserum is due to the reaction of individual molecular "species" in the antigen complex with their homologous antibodies. This assumption is not inconsistent with the observed behavior of antigen-antibody systems.

The serological identification of all proteins resides in their structural peculiarities and in their chemical nature, i.e., the kinds, proportions and arrangement of the amino acids and prosthetic groups all are believed to affect the serological activity of proteins. It is well known that mild treatment both chemical and physical, will alter the nature of proteins (Landsteiner, 1936; Cohn and Edsall, 1943). This fact presents a challenge to all investigators who are using animal proteins as representing the nature of the organisms with which he is working. Studies in serological systematics may require that animal sera be collected and saved, sometimes for a period of years, before numbers of different species sufficient for comparative in-

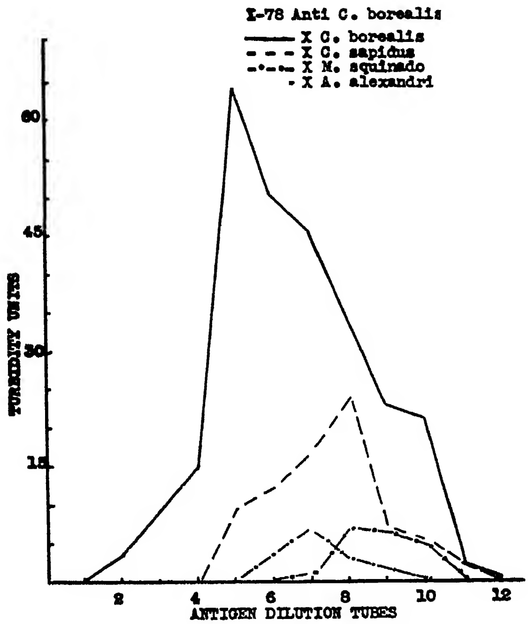

FIGURE 3. A typical series of precipitin titration curves showing an order of serological relationship among four crabs The homologous reaction between *Cancer borealis* and the antiserum produced against it exceeds all others. Then the alignment occurs, in the order of their decreasing curve areas, *Callinectes sapidus, Acanthocarpus alexandri,* and *Maia squinado,* which is in accord with accepted systematic relationships.

vestigations are secured. Further, sera frequently have to be collected under field conditions, necessitating the use of preservatives. There are other aspects of the general problem of antigen comparability than those resulting from the chemical action of preservatives which must also be considered before comparisons of animal sera can be made with confidence. Following are the results obtained from attempts to test the affect of some of the variables encountered in the preparation of animal sera for comparative studies in serological systematics.

Physical treatment

Usual laboratory procedures have been to allow the blood to clot and the sera to be expressed for about 24 hours. The sera are then centrifuged at 2000–3000 r.p.m. for 20 minutes, filtered sterile in Seitz filters, bottled, and stored in the refrigerator at 3° ± 1° C. Any or all of these steps could alter the nature of the serum proteins and thus modify their serological activity. Table III summarizes a series of tests performed to examine the effects of treatment in the laboratory. The sample of blue crab serum designated in the table as *Callinectes sapidus* 47–2 represents the sera of 24 large male crabs all bled within one hour. The pooled collection of sera was divided into several parts and given various kinds of physical treatment in the laboratory. All of the antigens were tested against antiserum I–86 (Anti-*C. sapidus* 47–1) which was prepared against a fresh, sterile filtered

sample of crab serum. The antiserum I–86 was powerful and was diluted with 10 parts buffered saline at pH 7.0. Since dilution is known to increase the specificity of an antiserum (Boyden and DeFalco, 1943) under certain conditions it was advantageous to dilute the antiserum in this manner to magnify any differences among the antigens. The dilution factor was so chosen that total turbidities would summate in the vicinity of 300 galvanometer units. A curve area this large tends to minimize variations in results due to errors in experimental techniques. Experimental error is limited to 5 per cent. In addition to the treatments listed in the table attempts were made to lyophilize (freeze-dry) (Florsdorf and Mudd, 1935) parts of this sample, but "boiling" [3] occurred during the course of drying, which denatured the proteins to such an extent that insufficient amounts of them could be restored to conduct comparative studies.

TABLE III

Effect of laboratory handling on antigen reactivity

Antiserum I–86 Anti *C. sapidus* 47–1 (1 + 10) × *C. sapidus* 47–2

Treatment	Area	% Change
Centrifuged, filtered	279	0
Centrifuged, unfiltered	283	1
Uncentrifuged, unfiltered	287	3
Frozen, centrifuged	287	3
Frozen, uncentrifuged	290	4
Room temperature, centrifuged, filtered	275	1
Room temperature, centrifuged, merthiolate	285	2

The homologous antigen *Callinectes sapidus* 47–2 was centrifuged and sterile filtered before it was used as an antigen. Changes due to above listed treatments appear to be negligible.

It is realized that any tests involving comparisons with only a single antiserum prepared from but one kind of the possible antigen types present but a minimum of data on the comparability of antigens. Moreover, in the type chosen (Table III, explanation), it still is possible that the unfiltered, and the frozen samples could have constituents not possessed by the filtered samples. That there was a high degree of correspondence among all of the antigens tested is testimony to the fact that the unfiltered, and frozen samples contain corresponding antigens to those in the filtered material, also that filtration does not significantly reduce the quantity of such antigens nor the quality of their reacting (combining) capacity. For the short period of time (24 hours) involved between the beginning and end of the processing, the sample held at room temperature showed no difference from the others. A retesting of the room temperature sample seven days later also revealed no significant change in activity.

[3] This is the bubbling which occurs when the rate of sublimation of the frozen material undergoing desiccation is slow and thawing occurs at the inside glass surface of the containers because of the transfer of atmospheric heat to the frozen solid while the whole system is under vacuum. The relationships between the rate of heat intake from the atmosphere at the exterior glass surface of the containers, the rate of heat loss at the evaporating surfaces of the product and the rate of escape of water vapor from the product to the condenser is apparently upset.

Effect of cold storage

The question of antigen comparability is bound to the problems related to the storage of sera for prolonged periods. In order to examine adequately the effects of storage it was necessary to establish whether or not significant alterations occurred in samples of sera of various ages. Table IV summarizes the results of these tests. It is readily seen that no demonstrable immunological alteration occurred between the samples one day old (22 hours) and the other older samples.

TABLE IV

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation	Age of sample when tested
47–3	12	7.04	280.1	−0.3	22 hours
47–2	12	7.00	278.8	−0.1	1 month
[b]47–1	24	7.39	279.1	0 0	4 months
36–1	5	5.05	281.8	−0.9	11 years

Table showing the lack of change in serum samples of the blue crab, *Callinectes sapidus*, when tested with an antiserum against one of them ([b]). Samples stored at 3° ± 1° C.

The fact that the eleven year old sample number 36–1 of *C. sapidus* possessed a precipitinogen activity equal to the very fresh sera of this species is very surprising and important. This old serum has been kept at refrigerator temperatures practically continuously since it was collected in 1936. The amount of alteration that goes on in these serum proteins, if any occurs, must be very slight not to be detected by immunological testing. All the samples listed in Table IV represent pooled samples of the sera of five or more animals. It is conceivable, however, that individual variability of the sera does exist and that the pooling nullifies this variation, presenting for test a more or less "common denominator" kind of serum to the testing antiserum.

Serological variation

Interesting evidence of the stability and lack of variability among various samples of sera of the same species of spiny lobsters, *Panulirus argus*, is illustrated in Table V. Here samples collected over a period of years (1932–1939) were tested against an antiserum made to one of them. The test antigens were selected to reveal the differences due to aging, differences due to sex, and differences between single specimen samples and pooled samples containing the sera of more than one individual. The amount of difference in the serological reactivity among these different categories was negligible. In no instance did the variations in the whole curve areas exceed the experimental error of the method of testing. This is a remarkable fact, considering the length of time these sera have been stored; the youngest sample being nine years old at the time of testing, the oldest sample being fifteen years old.

Considering the sensitivity of the precipitin tests to slight alterations in chemical structure, the lack of variation in the amount and kind of reactivity demonstrated in the data of Tables IV and V is testimony first to the care in the laboratory prepa-

TABLE V

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation
32–1	"Pooled"	6.07	345.3	−0.2
32–2	"Pooled"	6.52	345.6	−0.1
34–C	1	4.55	351.6	1.5
34–D	1	5.33	340.8	−1.4
34–E	1	2.66	357.0	2.9
34–F	1	7.66	355.7	2.8
34–G	1	3.69	343.7	−0.6
36–1	1	5.31	345.2	−0.2
36–3 & 4	2	10.05	353.6	2.5
36–5 & 6	2	7.44	350.6	1.3
36–10	1	6.07	354.5	2.5
36–11	4	6.52	351.3	1.5
*39–1	5	4.59	345.9	0.0
39–3	9	6.02	349.4	0.2

Table showing the high degree of serological similarity among different samples of the spiny lobster, *Panulirus argus* collected between 1932 and 1939, and tested with an antiserum against one of them (*). Tests were conducted in 1947.

ration of the sera, and the use of a correct method for maintaining useful samples for long periods of time, and second, a testimony to the durability and stability of hemocyanin proteins when collected and preserved in the manner described above.

Color changes

Hemocyanin proteins possess a copper radical and in the oxidized state and in vitro have a blue to green color when in solution. Occasionally, variations have been noted in the color of the sera after they have been sterile filtered and bottled. The most frequent variation is a change from the blue-green color to dark brown. This change is due in part to the free carbon on the Seitz pads which forms there as a result of over-heating during sterilization of the filter, and also to chemical changes in the proteins themselves since the color change has been noted not only during the filtration processing but also in some samples previous to filtering. The brownish color does not appear to play any role in altering the reactivity of the sera since these samples compare favorably with others of the same species possessing the blue-green color.

If a sample of hemocyanin serum is contaminated with bacteria, these organisms flourish and the blue-green, oxidized condition of the serum changes to a colorless liquid, with a cloudy suspension of bacteria. If such sera are kept in the refrigerator, even though contaminated, no recognizable alteration occurs in the activity of the dissolved protein when tested against an antiserum. Removal of the bacteria by filtration or centrifugation or both quickly restores the oxidized condition of the serum. Its serological behavior seems not to be altered even when the serum has been stored in the contaminated state in the refrigerator for prolonged periods.

Precipitates in vials

Another phenomenon that has been seen to occur in sterile, bottled sera is the appearance of precipitates in the vials. In some instances the amount of material coming out of solution has been considerable. The supernatants of many of these vials were examined and found still to contain sufficient protein in solution to warrant testing for comparability with nonprecipitated samples of the same species. Surprisingly enough the still soluble fractions of the total proteins in the antigens apparently possessed all of the reactivity characteristic for the serum. This would indicate that the precipitate is denatured protein which has not undergone any appreciable decomposition. The presence of large amounts of free amino acids and peptides in solution above the precipitates would present free radicals that might combine with the antiserum, blocking the reaction and causing some difference in the amount of reaction observed to occur between the still soluble protein and the antiserum. This is not the case with these systems. The precipitates, moreover, still possess the capacity to combine with antisera, as saline suspensions of them readily reveal. The antigen precipitates go back into solution readily using dilute alkali, but not in saline solutions up to 1.7 per cent NaCl.

Serological Systematics of Some Crustacea

Using the procedures described above, it thus appears feasible to collect and store the sera of Crustacea for long periods of time (15 years in the data given) without significant alteration in their specific properties. Comparative studies of the various species of Crustacea become in effect analysis of the biochemical nature of the organisms concerned based on the quantitative comparison of the nature of their serum proteins, as reflected by turbidities developed in the antigen-antibody reaction.

Erhardt (1929) reviewed the early work done on serological systematics among the Crustacea. Some of these early investigators obtained results which do not agree with systematic classifications based on morphological and embryological data. In one instance Nuttall and Graham-Smith, each using the same antiserum, compared exactly the same organisms and obtained very different results. In most other instances the serological work generally agreed with well established classifications. Most all of these early tests were done using the interfacial or "ring" test method for comparisons. The correspondence of techniques among the various workers ended there because the antigens were not standardized and because results of tests based on antiserum titers and those based on antigen titers cannot be directly compared.

Boyden (1942) pointed out the deficiencies and inadequacies in these early serological investigations and in two papers (1939, 1943) presented the first really quantitative study in the serological systematics of the Crustacea. He compared representatives of five families of the Brachyura and two species of the Macrura. It was in the later paper that the concept of the "serological yardstick" was introduced. The relationships of the sera of species of the same genus, and genera of the same family, and different families of the tribe Brachyura were remarkably consistent in their serological values. All of his tests were made using the photronreflectometer and standard procedures as outlined by Boyden and De-

Falco (1943). The inconsistencies apparent in earlier investigations by other workers were not observed to occur in these studies.

The availability of a larger and more representative group of Crustacea made an extension of Boyden's work possible. Table VI summarizes the serological comparisons made. It should be pointed out that the values given are averaged from two or more tests. The generally higher interspecific, intergeneric and inter-family relationships than those reported by Boyden seem to be due to the use of the presensitization technique to produce more powerful and less discriminating antisera.

From Table VI it is apparent that the antisera differ in their capacities to discriminate among the families of Brachyura. It appears that the Portunidae, Xanthidae and Cancridae are more closely related to each other than they are to the Ocypodidae, Calappidae and the Majidae. Of special interest is the species *Geryon quinquedens* here listed as a member of the family Goneplacidae, in accordance with Rathbun's classification (1937). She concluded that Goneplacidae were closely related to the Xanthidae. Bouvier (1940) places Geryon in the family Xanthidae. The serological tests indicate a degree of correspondence for *Geryon* which is approximately the same in heterologous reactions as for the xanthid species. Further critical testing is necessary to definitely establish the affinities of *Geryon*.

TABLE VI

The relative relationship among Crustacean species representing seven families in the tribe Brachyura

Antisera	Antigens (Relationship in per cent)												
	Callinectes sapidus (Portunidae)	*Callinectes marginatus* (Portunidae)	*Carcinus maenas* (Portunidae)	*Cancer borealis* (Cancridae)	*Cancer irroratus* (Cancridae)	*Cancer pagurus* (Cancridae)	*Cancer anthonyi* (Cancridae)	*Panopeus herbstii* (Xanthidae)	*Menippe mercenaria* (Xanthidae)	*Ocypode albicans* (Ocypodidae)	*Acanthocarpus alexandri* (Calappidae)	*Geryon quinquedens* (Goneplacidae)	*Maia squinado* (Majidae)
Callinectes sapidus (Portunidae)	100	78	46	33	44	37	33	24	48	18	12	38	8
Cancer borealis (Cancridae)	29	22		100	75	54		20	42	9	8	22	9
Cancer irroratus (Cancridae)	49	46	33	78	100	73	72	30	35	43	10	31	30
Menippe mercenaria (Xanthidae)	27		21	24	21	16	24	37	100	8		27	3
Ocypode albicans (Ocypodidae)	30		40	33		34		39	34	100	39	6	33
Acanthocarpus alexandri (Calappidae)	12		13	15		15				18	100	14	2
Geryon quinquedens (Goneplacidae)	15			13					15		8	100	9
Maia squinado (Majidae)	21			16					13	13	8		100

From Table VI it is readily seen also that species within a given genus react with each other to a greater extent than with any other organisms.

The problem of establishing in detail interfamily relationships among Crustacea by serological methods is one that would entail the production of large numbers of antisera in an attempt to secure serological reagents which are sufficiently powerful to react significantly with the more distant families and at the same time discriminate among the representatives of these families sufficiently to establish a verifiable order of relationship.

Summary

Serological Systematics is a branch of Serology concerned primarily with the classification of organisms. The taxonomic characters usually concerned are the serum proteins of organisms. The natural relationships obtained are those revealed by an antiserum in combination with its homologous antigen and various heterologous antigens which react in proportion to their degrees of correspondence to the homologous antigen. Many factors influence the antigen-antibody reaction. A few conditions which are of importance to studies in systematic serology have been investigated and the results given.

Antigens tested for comparability under a variety of the circumstances met in ordinary laboratory handling such as freezing, filtration and centrifugation, showed no significant deviation from each other in their serological activity. Age, within the limits stated, was shown to have no effect on the serological activity of serum antigens which are sterile filtered and stored just above freezing. Antigens in cold storage for as long as 15 years had the same activity as freshly prepared samples. Pooled serum antigens showed no serological differences from the sera of individuals. No differences were demonstrated in sera due the sex of the organisms. For cold stored antigens color changes in the vials did not indicate alteration of the reactivity of the proteins. The remaining soluble portions of protein in vials showing apparently spontaneous precipitation of the protein gave the same reactivity as freshly prepared antigens. Bacterial contamination, if not permitted to endure too long, and if kept under refrigeration, does not alter the proteins significantly. The reconstitution of lyophilized hemocyanin sera for serological testing was not successful.

The studies in systematic serology have been extended to include new families of decapod Crustacea. For the species of Brachyura tested it appears that the families Portunidae, Xanthidae, and Cancridae are more closely related to each other than all of them are to the Ocypodidae, Calappidae and Majidae.

Further studies into the conditions which may modify the serological reactivity of proteins are needed. Continuing investigations, examining both the validity of the methods used in measuring antigen-antibody reactions and the methods used in the preparation of these primary serological reagents, are necessary components of truly critical studies in serological systematics.

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THE GROWTH AND METAMORPHOSIS OF THE ARBACIA PUNCTULATA PLUTEUS, AND LATE DEVELOPMENT OF THE WHITE HALVES OF CENTRIFUGED EGGS

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The pluteus of *Arbacia punctulata* which is well known to many investigators is the early pluteus with four arms, a pair of long anal (post-oral) arms on the ventral side, and a pair of short oral (pre-oral) arms on the dorsal side. In order to obtain further development of the pluteus, it is necessary, at Woods Hole, to feed the animals a rather special diet. In 1882 W. K. Brooks and two of his students, Garman and Colton, raised the plutei of *Arbacia punctulata* at Beaufort, N. Carolina, apparently without any special feeding. The sea water there is rich in diatoms, and the plutei can probably obtain what they require for growth from the sea water. This work was published by Brooks (1882) in his Handbook of Invertebrate Zoology, and by Garman and Colton (1883). The drawings are excellent for detail, but there is no indication of size and not adequate indication of age. A few stages had previously been described and figured by Fewkes (1881) working in A. Agassiz's laboratory at Newport, R. I. The present account with photographs gives the development of the pluteus with regard to size, sequence of events and rate of development at Woods Hole.

The best food for sea urchin larvae has been found to be the diatom, *Nitzschia closterium;* I found that they would also grow on the diatom, Licmophora. The Nitzschias themselves require a special diet, and must be raised in pure culture; they are raised on Miquel's solution.[1] The method has been worked out by Allen and Nelson (1910) in Plymouth, England, and has been used by many investigators at the Plymouth laboratory. Shearer, deMorgan and Fuchs (1914) have in this way succeeded not only in raising the normal plutei of several species of sea urchin to maturity, but have also raised some hybrid plutei to maturity. Fuchs (1914) has even obtained the next or F_2 generation of these hybrids. Unfortunately, the late

[1] Miquel's solution, as modified by Allen and Nelson (1910), consists of:

Solution A	KNO_3	20.2 grams
	Distilled water	100 cc.
Solution B	$Na_2HPO_4 \cdot 12\ H_2O$	4 grams
	$CaCl_2 \cdot 6\ H_2O$	4 grams
	$FeCl_3$ (melted)	2 cc.
	HCl (concentrated)	2 cc.
	Distilled water	80 cc.

To each liter of sea water add 2 cc. Solution A and 1 cc. Solution B, and sterilize by heating to 70° C. When cool, decant off the clear liquid from the precipitate, which will have formed when Solution B is added to the sea water.

Ketchum and Redfield (1938) have used a slight modification.

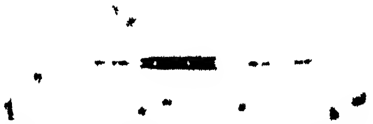
larval characters of *Echinus esculentus* × *E. acutus* from which the F_2 generation was obtained are alike in the two species, so that no information as to inheritance could be obtained, and none of the F_2 hybrids between *E. esculentus* or *E. acutus* × *E. miliaris* which would have given the information, reached maturity. Miss Gordon from MacBride's laboratory raised some Arbacia plutei at Woods Hole in 1926, using this method, but she was particularly interested in the later development of the test, and gives no account of the changes in the pluteus in her publication (1929).

There are two forms of *Nitzschia closterium* both of which are devoured by the plutei. One, the large form (Plate I, Photograph 1) is about 100 μ long; the other, forma *minutissima* (Photograph 2), is about 24 μ long and is the variety used in the Plymouth laboratory. The Nitzschias are swept into the oesophagus and stomach (Photographs 3, 4) by cilia.

Several stages in the early development from the fertilized egg are shown on Plate II (Photographs 1–6). The micromere stage (Photograph 2) is the first sign of differentiation of cells, the micromeres being small and colorless. It also marks the beginning of asynchronous cleavage, in Arbacia there is a definite 12-cell stage preceding the 16-cell stage. With further cleavages a blastula is formed and emerges from the fertilization membrane (Photograph 3) in about 8 hours, the time varying by one or two hours in different batches and with different temperatures. At this stage I have estimated that there are 1,000 to 2,000 cells representing 10 to 11 cleavages (2^{10} to 2^{11}). The blastocoel becomes larger, leaving a single layer of peripheral, ciliated cells (Photograph 4) Then invagination takes place (Photograph 5), and a gastrula is formed (Photograph 6). At this time the skeleton appears in the form of triradiate spicules, one on each side of the gut. During this period there is no appreciable increase in size of the organism over that of the egg (without the fertilization membrane), and one would not expect an increase before the alimentary canal is complete and it can take in food from the outside. Now growth occurs and differentiation into the pluteus form with skeletal rods on each side (Photograph 7) At this stage the large pigment spots characteristic of the later plutei begin to form The young pluteus increases in size and the arms begin to grow out (Photograph 8) The pluteus is well formed in 24 hours (Photograph 9). It is larger on the second day (Photograph 10), usually reaching a maximum in three or four days (Photograph 11) The long anal arms may measure 410 μ from base to tip. Without special feeding, the pluteus may live three or four weeks, gradually getting smaller by resorption of its arms (Photographs 12–14). It has apparently obtained sufficient food for growth from the sea water for the first four days, but then requires additional food. The structure of a two day pluteus is shown in serial photographs (Plate I, Photographs 5, 6, 7). These are taken at different levels through the animal, corresponding to serial sections of imbedded material

Further development of the three or four day pluteus may take place if *Nitzschia closterium* is added to the cultures of plutei. Only a few plutei in any culture continue to develop. The British investigators have found it expedient to have only a few individuals in a large amount of water, 20 to 30 in a half-gallon jar. (See MacBride 1914, p. 506.) I raised them in Syracuse watch glasses holding about 15 cc. of sea water with about a dozen plutei in each dish. The developing larvae

PLATE I



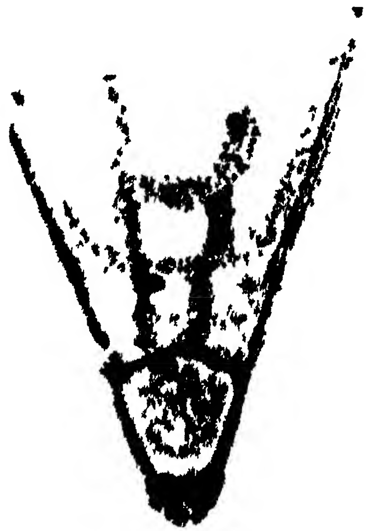
1

Nitzschia closterium

2

Nitzschia closterium
forma minutissima

3



4

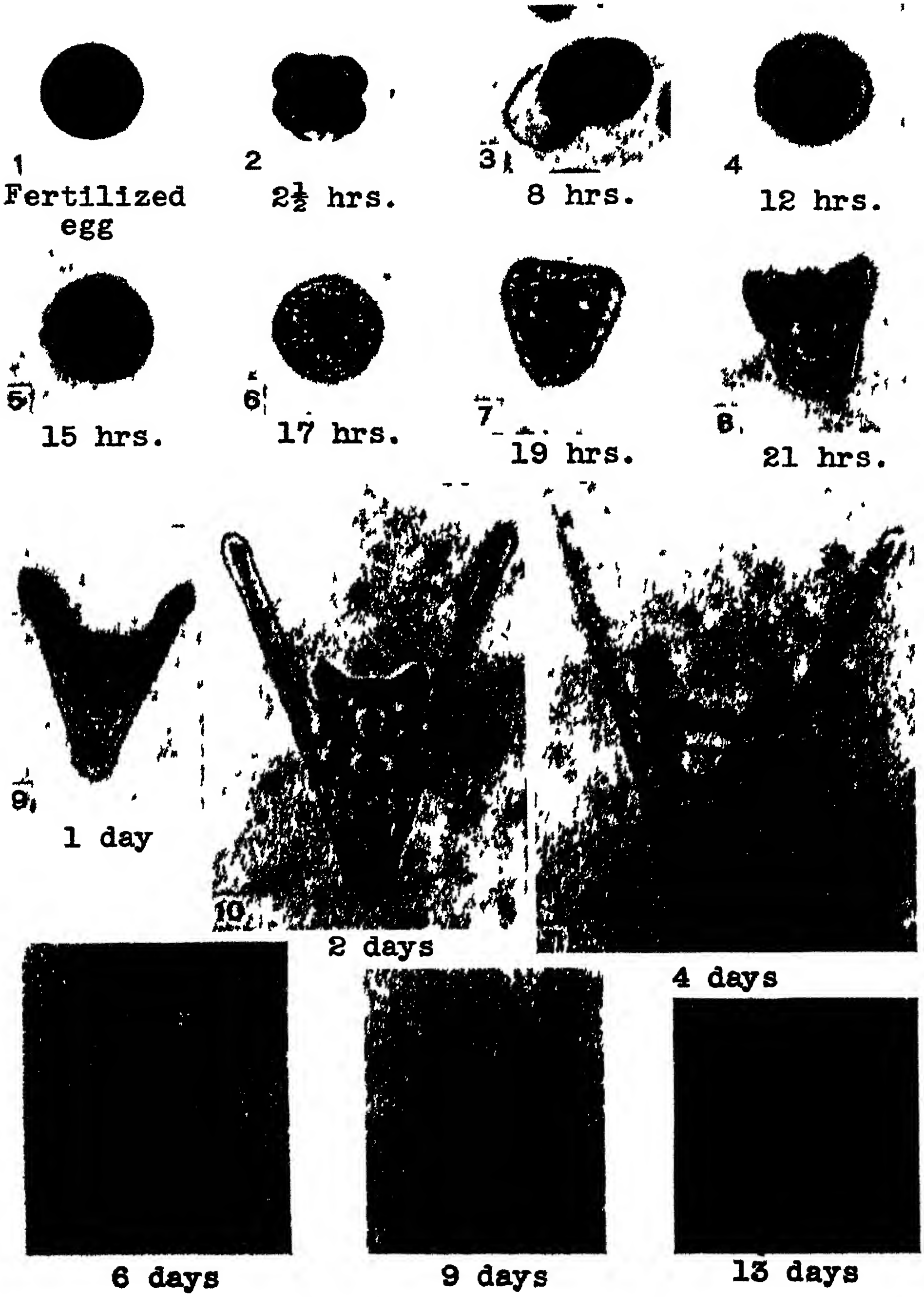
Plutei engulfing Nitzschias

Serial photographs of 2 day pluteus

Dorsal Median Ventral

PLATE II

DEVELOPMENT WHEN NOT FED



were transferred to fresh sea water with a pipetteful of the Nitzschia culture every few days. The eggs from which the final small adults were obtained were fertilized on July 12th, 1948, at Woods Hole, and taken to Princeton, N. J, on October 3rd. The last four died after completing metamorphosis on November 17th, a little over four months old.

The photographs on Plates III and IV, 1–18 are all to the same scale The development varies in time in different batches, and in individuals of the same batch, so that the times given are only approximate The eggs at this magnification (approximately 24 times) are of the size shown in Photograph 1. The one and the three day plutei to this scale are shown in Photographs 2, 3, shown with a larger magnification in Photographs 9, 10, 11 on Plate II (approximately 180 times). For the first four days, the development is the same whether fed Nitzschia or not, the food in the sea water being adequate. When fed, in about a week (Photograph 4, Plate III), the anal arms have become considerably longer. Then little knobs appear toward the base of the animal, which, by the eleventh day have grown out into a definite pair of new arms, the ventral lateral or postero-lateral (Photograph 5). These are always heavily pigmented at the tips which appear very red These grow longer and a pair of knobs appear between the original anal arms and the new red-tipped arms. These are slightly noticeable in Photograph 6. In a month's time, these have grown into a second new pair of arms (Photograph 7); these are the dorsal lateral or postero-dorsal arms These arms usually do not have red tips, although sometimes all the arms are pigmented at the tips All the arms become continually longer (Photograph 8). The animals are now easily visible to the naked eye and look like small spiders. The arms are variable in length individually and relatively to each other. They are all ciliated, the cilia on the red-tipped ones being much longer and stronger than those on the other arms. The animal now swims actively by means of its cilia, and also walks or tumbles about on the tips of its arms, which can be readily moved. The arms are quite fragile and are easily broken off when the animal bumps into something or when it is transferred to another dish. They have great regenerative capacity, the arms growing out again when broken off One pluteus, from which I had cut off the red-tipped arm about half way down, had completely regenerated it together with the red pigment in five days so that it then looked exactly like its mate. The broken piece may seal itself off and swim about actively by means of its cilia like a complete organism, looking something like a paramoecium (Plate VI, Photograph 6). One of these pieces was alive and active for four days when it was inadvertently lost. The body of the adult Arbacia is seen as a yellowish green mass in the pluteus, the dark area in Photographs 7, 8 (Plate III) and thereafter. There are areas of dark red pigment on the surface. The young adult is formed in the body of the pluteus and grows at the expense of the pluteus.

By six weeks the pluteus has become quite complicated, with two pairs of secondary oral arms, and four tubular processes (auricular lobes), two dorsal and two ventral. In Photograph 9, one sees several of the oral arms (at the anterior end) and a pair of the tubular processes (at the posterior end). A diagram of this stage is given by Miss Gordon (1929, p. 291). Soon after this, the five primitive ambulacral feet appear at one side of the body; these have suckers at their extremities and are continually expanded and contracted (Photograph 10).

PLATE III

DEVELOPMENT WHEN FED

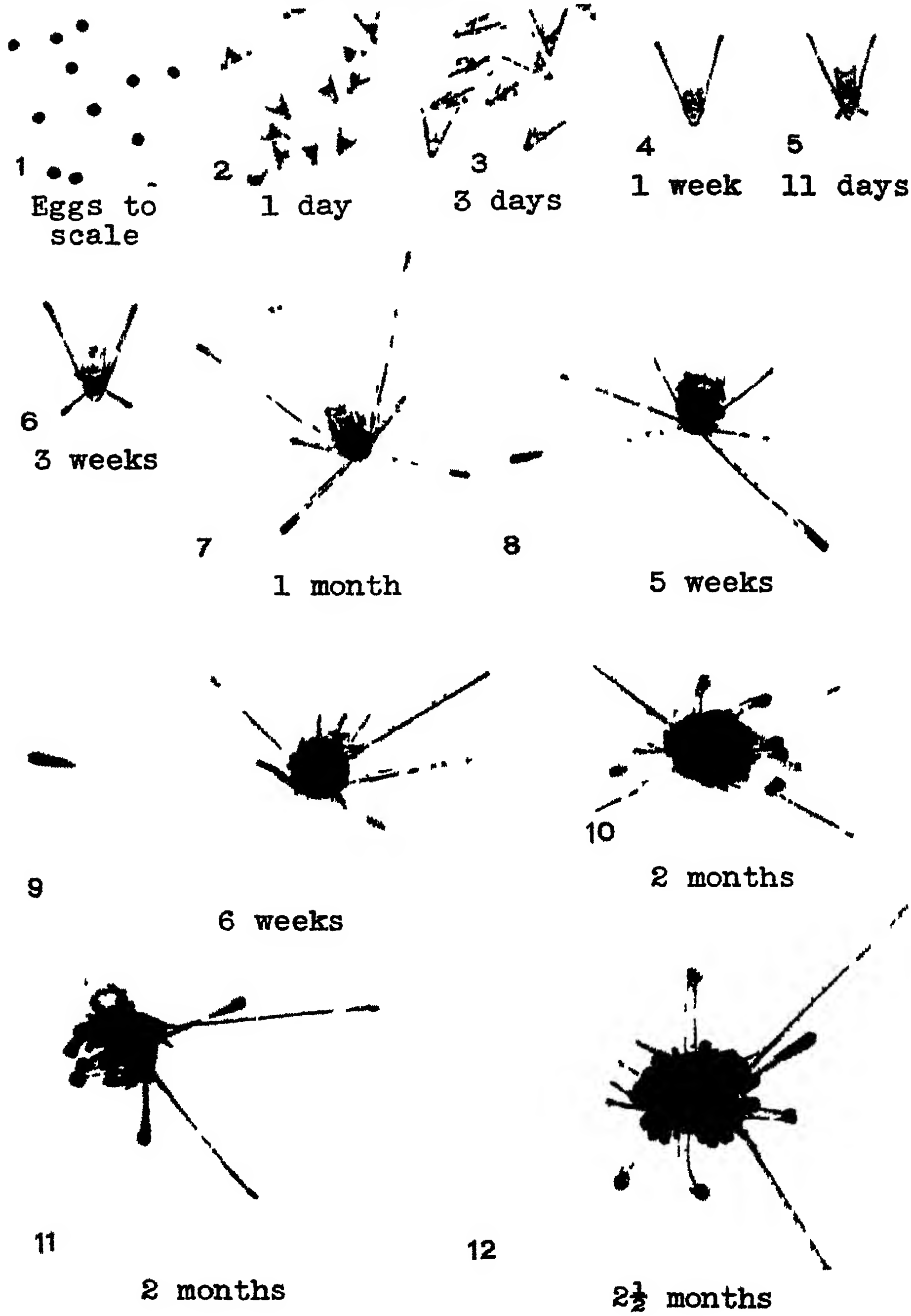
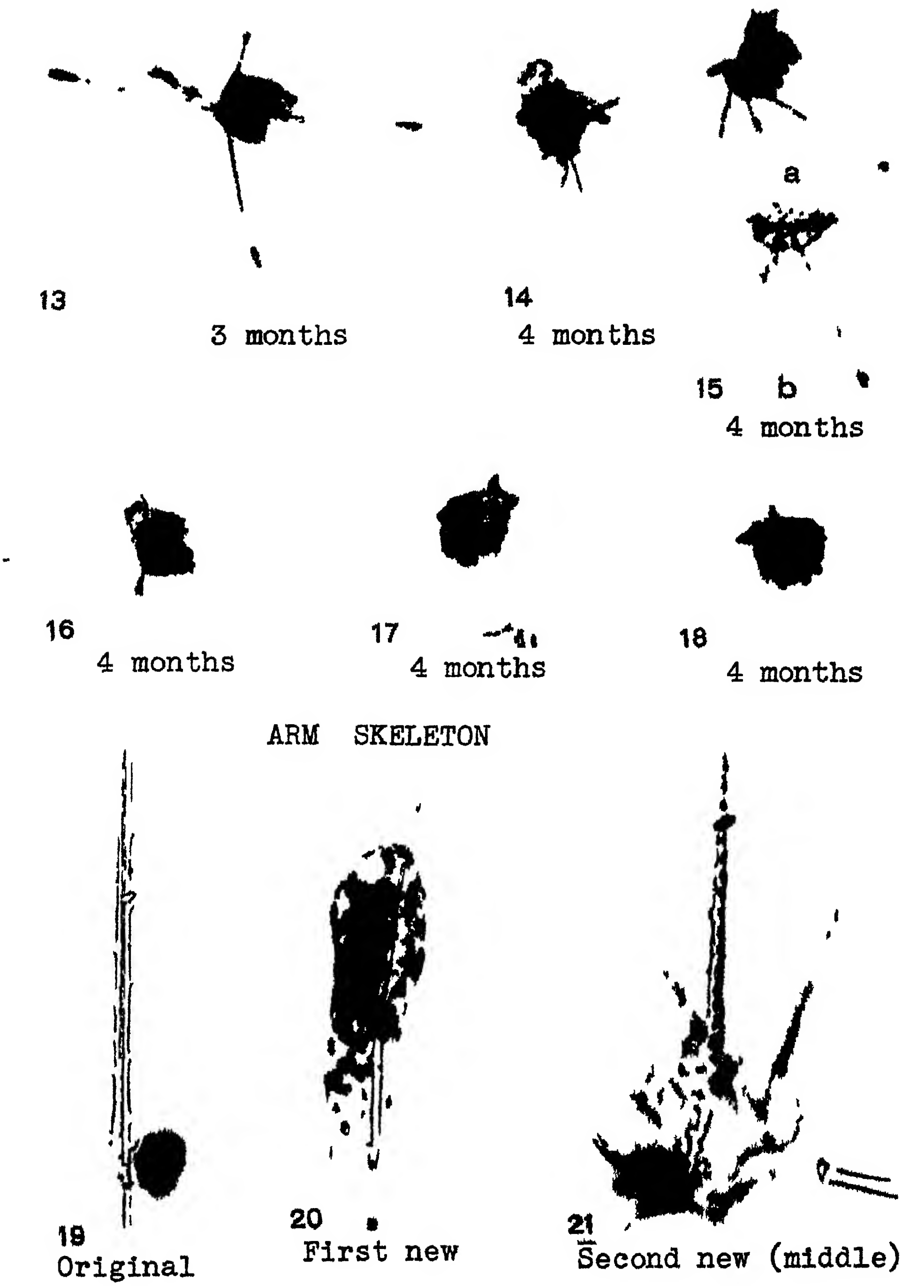


PLATE IV

DEVELOPMENT WHEN FED II



In Photograph 11, also a two months pluteus, one sees the three pairs of long arms, two pairs of the short arms near the head of the pluteus which is still prominent, the five ambulacral feet, with suckers, and the body of the developing adult. After two or more weeks, between each two ambulacral feet are formed three flattened plates, the first set of spines (Photograph 12). The pluteus has now reached its full development and the arms their maximal length, ca. 1 6 mm. The length of the arms is quite variable in different individuals even of the same batch To give some idea of the increase in length of the arms, the following table is given of the length of the long anal arm at different ages. The figures represent measurements of the average better developed ones, the poorly developed ones not being taken into account

Approximate length of long (anal) arm from base to tip (μ)

	Fed	Not fed
1 day	180	180
2 day	300	300
3 day	380	380
4 day	410	410
5 day	450	330
6 day	480	250
1 week	600	200
11 days	700	180
2 weeks	750	150
3 weeks	800	
1 month	1000	
1½ months	1300	
2 months	1400	
2½ months	1600	

After reaching their maximal size, and often earlier, the arms begin to go to pieces; the flesh peels off, leaving the bare skeleton (Plate IV, Photograph 13). By four months several of the arms have gone to pieces; the body of the adult is conspicuous, but the head of the pluteus remains (Photograph 14). Sometimes the arms are shed as a whole piece like a shell. In Photograph 15 three of the arms shown in the upper Photograph (a) were shed as a unit the following day, as shown in the lower Photograph (b). The animal was left with one arm (Photograph 16). This was thrown off, but the head of the pluteus remained, and the body of the adult with its primitive spines was developing (Photographs 17, 18). The animal at this time was about a half millimeter in diameter. These later stages in metamorphosis took place in my cultures within a few days. The whole process from fertilization to metamorphosis took over four months (July 12 to November 17). I do not know whether this is the normal period under natural conditions as my cultures were subjected to changes in temperature and food. My last four animals all died at this time. They are known to require a different food after metamorphosis, which I did not have at hand. According to Shearer, deMorgan and Fuchs (1914), the best food is the calcareous protozoan Trichosphaerium; and later the red alga Corallina, these furnishing the calcareous matter needed for the development of the test and spines (l.c., p. 276). Miss Gordon (1929) has given a complete account of the further development of the young adult with especial attention to the test.

PLATE V

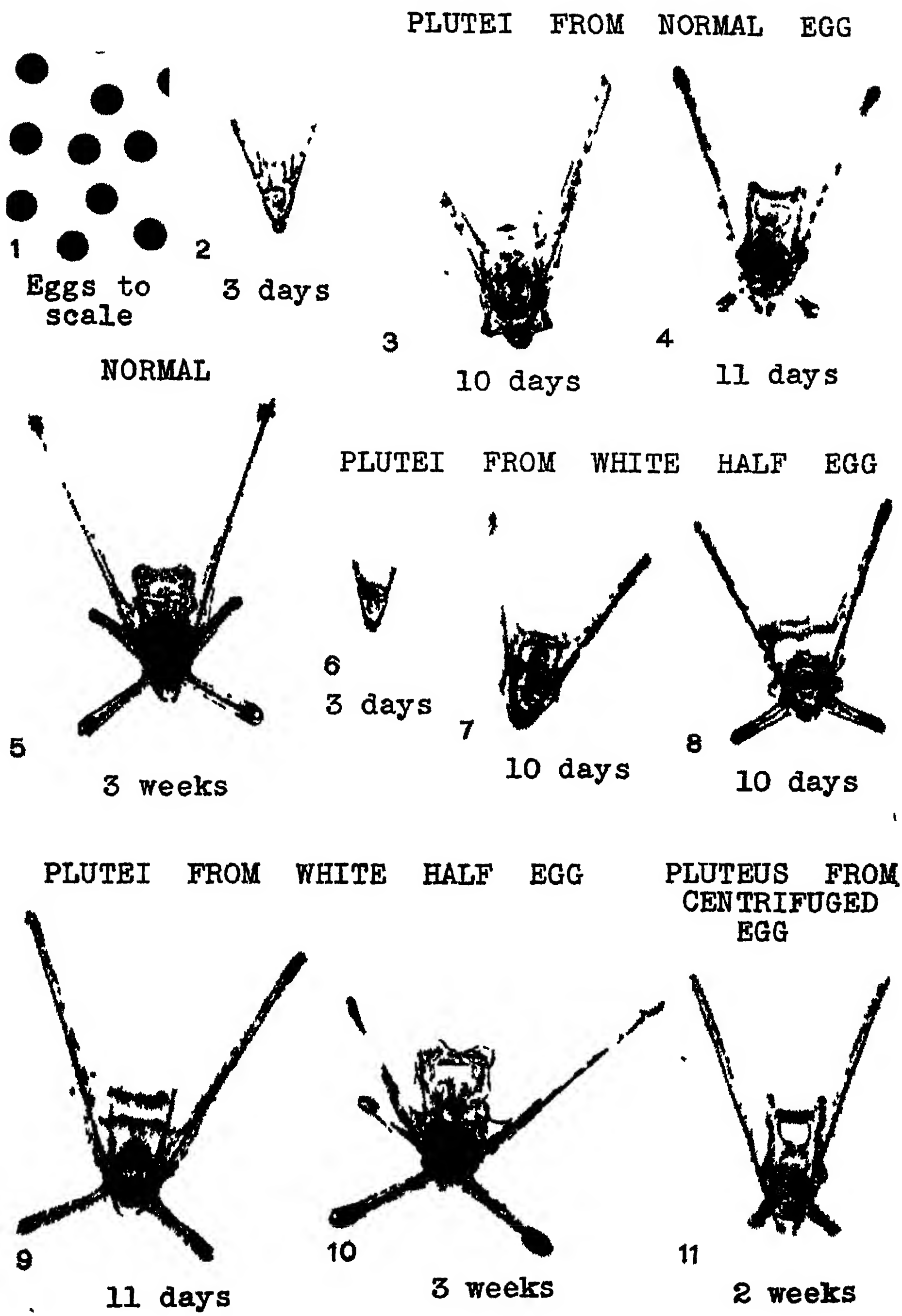
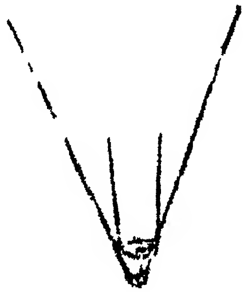

PLUTEI FROM NORMAL EGG
1
Eggs to scale
2
3 days
3
10 days
4
11 days
NORMAL
5
3 weeks
PLUTEI FROM WHITE HALF EGG
6
3 days
7
10 days
8
10 days
PLUTEI FROM WHITE HALF EGG
PLUTEUS FROM CENTRIFUGED EGG
9
11 days
10
3 weeks
11
2 weeks

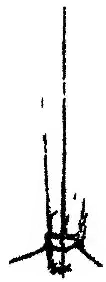
PLATE VI

SKELETONS

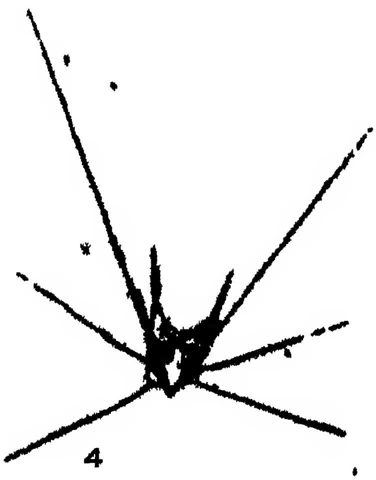
1
2 days



2
8 days



3
11 days



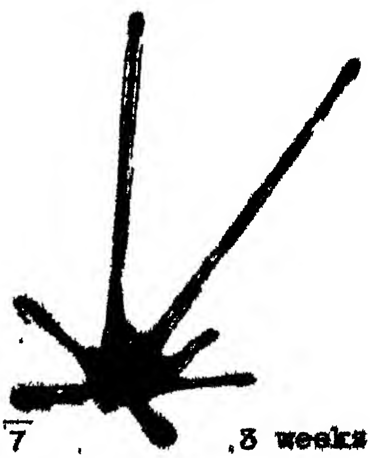
4
1 month

5
White half

ABNORMAL

BROKEN OFF
ARM

6



7
3 weeks

The arm skeletons of the pluteus are interesting. The skeleton of the original long pair of (anal) arms is fenestrate, that is, it is a rod with holes in it (Plate IV, Photograph 19). The new pair of red-tipped (ventral lateral) arms have a solid skeletal rod with no holes (Photograph 20). This photograph shows also the concentration of pigment at the tip. The rod in the second new pair of long (dorsal lateral) arms, between the other two long pairs, is again fenestrate (Photograph 21). As is well known, the skeletons of the original long (anal) arms are solid in the plutei of many sea urchins. We have, in general, two types of plutei, those with fenestrate arm skeleton such as Arbacia, Tripneustes, Sphaerechinus and the sand-dollar, Echinarachnius; and those with solid arm skeleton, such as Strongylocentrotus, Lytechinus and Psammechinus. This has been of great value in hybridizing experiments, in determining maternal and paternal inheritance.

Development of the white half egg

The Arbacia egg can be separated into a white and a red half by centrifugal force. The white half when fertilized develops in the same way as the normal whole egg (E. B. Harvey, 1932, 1940). A normal pluteus is formed in two days similar to that from the whole egg except that it is smaller and lacks pigment. The pigment spots, however, begin to come in on the third day after fertilization, and continually increase. The white plutei, like those from the whole egg, do not develop beyond the four armed stage without special feeding. The photographs on Plate V were taken at a greater magnification than the preceding series on Plates III and IV (i.e. approximately 60 times). The eggs to scale are shown in Photograph 1; the normal three day pluteus in Photograph 2. Several later stages of normal plutei are shown in Photographs 3, 4, 5, for comparison with the same stages of the white plutei (Photographs 6–10). The ten day white plutei (Photographs 7, 8) are in all respects like those from whole eggs (Photographs 3, 4). There is the same massing of red pigment in the tips of the first new pair of ventral lateral arms. There is now no difference in size between the plutei from the half egg and those from the whole egg, in fact the former may be larger (Photograph 9). The three weeks pluteus from the half egg (Photograph 10) has the three pairs of long arms like that from the whole egg (Photograph 5). These were not carried any further, but it seems certain that the later development would be like that of the pluteus from the whole egg.

Development of the centrifuged egg

The very young pluteus from the centrifuged egg has the pigment granules concentrated in certain areas, most frequently above the mouth, though they may be in other positions. The pigment spots are also unevenly distributed at first, but after three or four days they are fairly uniformly distributed so that one cannot distinguish between the plutei from centrifuged eggs and those from normal eggs.[2] The later

[2] The pigment spots of the plutei are bright red, in contrast to the brownish red color of the unfertilized eggs. However, in centrifuged eggs, the concentrated mass of pigment granules is bright red. This difference in color is well shown in kodochrome slides when the contrasting objects are taken on the same slide. E. G. Ball (*Biol. Bull.*, 97: 231) has compared the absorption spectra of acid alcohol extracts of plutei and eggs, and has found the two pigments identical.

development, when fed, is like that of the normal pluteus. In Photograph 11 is shown a two weeks pluteus from a centrifuged egg, which is similar to the pluteus from a normal egg of about the same age shown in Photograph 4.

Skeletons

The skeletons from plutei of various ages are shown in the photographs on Plate VI; the fully formed skeleton is shown in Photograph 4. A skeleton from a white half egg is shown in Photograph 5, quite like that from a whole egg of the same age (Photograph 3).

Abnormalities

The only abnormal later pluteus occurring in my cultures is shown in Photograph 7, on Plate VI. It had one extra arm on one side.

Magnification of Photographs

The photographs were taken of the living animal with different objectives, as indicated below in brackets, and a 10X ocular.

Plate I. Photographs 1, 2 about 420X (70X). Photographs 3, 4 about 240X (40X). Photographs 5, 6, 7 about 180X (30X).

Plate II. All photographs about 180X (30X).

Plate III. All about 24X (4X).

Plate IV. Photographs 13–18 about 24X (4X). Photographs 19–21 about 240X (40X).

Plate V. All about 60X (10X).

Plate VI. Photographs 1–5 and 7 about 60X (10X). Photograph 6 about 240X (40X).

Explanations of the plates are given on the plates, and in the text.

Summary

1. The growth and metamorphosis of the *Arbacia punctulata* pluteus after the four day stage has been traced by means of photographs. The plutei were raised on the diatom, *Nitzschia closterium*.

2. The pluteus from the white half egg develops pigment spots after the third day, and develops in exactly the same way as that from the whole egg, when fed Nitzschia.

3. The pluteus from the centrifuged, stratified, egg becomes like that from the normal egg after the third day and develops similarly when fed.

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A NOTE ON THE REORIENTATION WITHIN THE SPINDLE OF THE SEX TRIVALENT IN A MANTID

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In most mantids during the formation of the spindle in late meiotic prophase, the kinetochores of each bivalent move suddenly apart towards opposite poles stretching the chromosomes. This is known as the premetaphase stretch stage and is followed first by recontraction of the chromosomes and only then by their gradual congression at the equator to form the metaphase plate. The bivalents move to the equator with no change in their original orientation of one kinetochore to each pole. The behaviour of the sex trivalents, however, both during the stretch stage and the ensuing congression, presents a more complicated situation. As normal behaviour one would expect the X_1X_2 kinetochores to orient towards one pole and the Y kinetochore towards the other, but actually a large number of sex trivalents appear to orient at random. There result, in addition to normal orientation, several types of malorientation. White (1941) first observed such malorientation of sex trivalents; he noted a high frequency of malorientation in 3 species of mantids and concluded that some reorientation must take place before metaphase formation. The conclusive proof for such reorientation was given by Hughes-Schrader (1943), who demonstrated, in *Stagmomantis carolina*, a decrease in the number of maloriented sex trivalents between premetaphase stretch and final metaphase. In the present note are recorded observations on *Hierodula* sp. which show that in this species also reorientation of trivalents takes place.

The material consists of testes fixed in PFA3, from a nymph of *Hierodula* sp., collected near Bombay (India), and placed at my disposal by Professor J. J. Asana to whom I am greatly indebted. Sections ranging from 6 to 10 μ and stained in iron haematoxylin were used for the study.

The presence of a sex trivalent in this species of Hierodula (specific identification is not available) was recorded by Asana (1934) who also established the total number of chromosomes in the male as 27. Later Oguma (1946) recorded the same chromosome complement in the males of four species of Hierodula.

In the present material all sex trivalents in which a lateral view of the spindle is presented during the premetaphase stretch, and again during the metaphase were counted. Those trivalents whose position prevented a positive determination of their orientation were also recorded. During the stretch stage various types of orientation were found. In some cases one of the X's was oriented towards one pole while the other X and the Y were oriented towards the opposite pole; in others the Y was stretched between the two X's while in the rest the orientation was normal. These configurations, assumed by the sex trivalents during the premeta-

* This work was done in the Department of Zoology, Columbia University. I am greatly indebted to Professor Franz Schrader for giving me the facilities to work and to Dr. Sally Hughes-Schrader for suggesting this problem and her helpful criticism.

phase stretch, involve a genuine orientation of the kinetochores to the division center comparable to that ordinarily occurring at metaphase. This is shown not only by the position of the chromosomes and their attenuation at the kinetochores but also by the fact that chromosomal fibers are formed between the center and the kinetochore in both maloriented and normally oriented chromosomes.

TABLE I

Orientation of sex trivalent during premetaphase stretch and at metaphase

	Normal	Malorientation			Not analyzable
	$X_1X_2 \updownarrow Y$	$X_1 \updownarrow Y \updownarrow X_2$	$X_1Y \updownarrow X_2$ $X_2Y \updownarrow X_1$	Total	
Premetaphase	42 =65.6 per cent	15	7	22 =34.4 per cent	36
Metaphase	149 =97.4 per cent	3	1	4 =2.6 per cent	0

It will be seen from Table I that during premetaphase stretch as many as 34.4 per cent of all analyzable sex trivalents are maloriented. This number is, however, strikingly reduced to 2.6 per cent in metaphase. Even if all non-analyzable trivalents were assumed to be normally oriented, still the number of maloriented sex trivalents is 22.0 per cent of the total, which is quite significant in relation to the 2.6 per cent of malorientation found at metaphase. This clearly proves the occurrence of reorientation in Hierodula and supports the earlier observations of White and Hughes-Schrader. This conclusion leads us to the basic question of what processes underlie the reorientation. The probable explanation must be sought in the role of the kinetochore. Further investigation of this problem in other species of mantids is planned.

SUMMARY

During the stretch stage in the meiosis of the male Hierodula a high percentage of malorientation of the sex trivalent is found. At metaphase, however, the number of maloriented configurations is so small that a considerable amount of reorientation must occur between these two phases. The forces involved are obviously of some significance in the general problem of the mitotic mechanism.

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MODIFICATION OF THE RESPONSES OF TWO SPECIES OF BUGULA LARVAE FROM WOODS HOLE TO LIGHT AND GRAVITY: ECOLOGICAL ASPECTS OF THE BEHAVIOR OF BUGULA LARVAE

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It is well known that the distribution of sessile organisms is markedly influenced by environmental conditions that affect the setting of their larvae. Thus, oysters are abundant at the mouths of certain rivers where their copper-laden waters are mingled with that of the ocean, and both maxima and minima of settings can be correlated with the amount of copper present during a critical stage of their development. (Cf. Prytherch, 1934.) Furthermore, rather frequent cases of co-occupation of a habitat by communities of sessile organisms of entirely different phyla would suggest the possibility that conditions favorable for the setting of one group are also advantageous to the other. The following observations on the effects of light and temperature on two species of *Bugula*, *B. flabellata* and *B. turrita,* from the Woods Hole region are presented partly because these factors influence distribution by affecting the attachment, metamorphosis and growth of the larvae and partly for the purpose of comparing and contrasting the behavior of these organisms with that of *B. neritina*, formerly studied at Beaufort, North Carolina (Lynch, 1947). The problem of distribution of two species of the same genus is often a baffling one, as is well illustrated by the fact that *B. flabellata* and *B. turrita* occupy communicating waters not more than 100 yards from each other yet each is found almost exclusively in its own particular habitat. A better understanding of the physiology of the larvae may lead to the beginning of a solution of such problems of distribution.

Contrasting features of the larvae of B. flabellata and B. turrita

Since a description of the larva of *B. flabellata* and its reactions to light and gravity has been given by Grave (1930), only contrasting features of the two larvae or additional details of their behavior will be presented here. *B. flabellata*, the smaller of the two (average, 0.17 by 0.19 mm.), has ten or twelve flagella in its pyriform region and is devoid of light-reactive organs. *B. turrita* is larger (average, 0.19 by 0.20 mm.), has four or five long slender flagella and four brilliant-red, spherical eye-spots, two very close to the pyriform organ and two slightly larger ones located in the opposite hemisphere.[1] The whole body of the larva, except the eye-spots fluoresces faintly in ultra-violet light of 3600 Å. Ejected hold-fast material and disintegrated larvae, however, do not fluoresce. This response to

[1] It is difficult to understand why Grave (1930) referred to the light-receptive organs of the larva of *B. turrita* as being "darkly pigmented" and failed to mention their red color. Even with the light cut down to a minimum, their brilliance is an outstanding feature.

ultra-violet light seems to be caused by some substance in the integument of the larva. The cilia at the equator of both species are more active than those in other regions.

The taxonomy of the genus *Bugula* leaves much to be desired at the present time. In the position, color and shape of the eye-spots and in external structure the larva of American *B. turrita* is identical with that of the European *B. plumosa,* but the ground color of the former is yellow or flesh-colored (like that of the European *B. flabellata*), with a faint band of orange pigment at the equator, whereas that of *B. plumosa,* according to Nitsche (1870), is pure white. Furthermore, the larva of European *B. flabellata* differs from the American form, devoid of light receptive organs, since the former has ten symmetrically arranged eye-spots that are figured by Nitsche (1870) as slit-like or elongated and surrounded by fine cilia, but are ovoid according to Barrois' (1877) plates. Grave (1930) noted the difference between American and European species and stated that Calvét (1900) also referred to a confusion of varieties. Is the organism called *B. turrita* in America merely a variety of *B. plumosa?* Or is this a case in which evolutionary changes have affected only the larvae in some instances and only the adults in others? Since two species of *Bugula* sometimes have identical larvae and since the same species apparently may have two different larval forms, it is not unreasonable to suppose that mutations could affect the form of either larva or adult independently of each other.

Materials and Methods

Adult colonies were kept in darkness over night and until the experiments were begun. After exposure of the parental colonies to light, the photopositive larvae released by illumination were easily pipetted to experimental vessels. Generally a single group of adult colonies yielded enough larvae for experiments on several different days. For observations on the geotropism of the larvae and their reactions to light, small vials 1.5 cm. in diameter and 8 cm. high, as well as stender dishes were employed; for microscopic examinations stender dishes and occasionally well slides were used. When slides were used, they were covered to prevent evaporation, which hastens setting by increasing the salinity. A box, 5 by 5 by 15 cm., covered with black paper on all surfaces except the exposed side, was used for experiments on the effects of colored lights.

The Reactions of the Larvae to Light, Heat and Gravity

Diffuse daylight. As described by Grave (1930), the larvae are intensely photopositive during the first three or four hours after their release from the ovicells and then become photonegative. The writer found the photonegative reaction to be somewhat more intense than that described by Grave (1930), who concluded that "it might be overlooked" because of its gradual onset. By 6–8 hours both species of larvae were always definitely photonegative and this reaction was intensified by placing them in sea water diluted by 50 per cent. Furthermore, they changed their reaction from positive to negative immediately after they were placed in sea water buffered to a pH of 9.6 (borate buffer) or diluted by 50 per cent, even when the latter was made hypertonic by the addition of sucrose. In sea water containing 2.5 mg. of copper chloride per liter the majority of the larvae, still photopositive 30

minutes after exposure, did not swim towards the light when the dish was reversed as they normally do. When a concentration of 5 mg. copper per liter was used, they became photonegative within 30 minutes. Even 0.5 mg. per liter reduced the intensity of the photopositive reactions. Since these peculiarities were not observed when the organisms were ejected from a pipette into sea water, mechanical force can hardly be the cause of this reaction. The absence of photic responses in mixtures of sea water and magnesium chloride was mentioned in a previous paper (Lynch, 1949).

Reactions to blue and red light. When a prism spectrum was used to illuminate vials placed horizontally in a black box about 16 feet from a 500 watt bulb, the larvae aggregated densely in the yellow, orange and red regions during their photopositive phase but afterwards congregated in large numbers (80–90 per cent) in the dark region beyond the violet when only the middle portion of the vials was illuminated, or in the violet region when the whole tube was exposed. Apparently this behavior was merely a response to heat, which the larvae tried to avoid, or to light intensity rather than to color.

Since the above method was unsatisfactory, the remaining experiments were carried out with blue and orange-red Eastman Kodak Wratten filters, numbers 76 and 72 respectively. Number 76 transmits a wavelength of approximately 4200–4800 Å and number 72 a wavelength of 5800–6600 Å. These filters have nearly equal relative energy transmission when illuminated with a 400 watt bulb according to Hecht (1921). One end of the vials was illuminated by blue light and the other by red. In the center there were two dark bands caused by the opaque paper of the edges of the two filters where they touched each other. More specimens attached in these dark regions than in either the blue or the red end; these, of course, were not counted in the total number that responded to the colored lights. Of the 292 larvae used in 5 trials 67 per cent attached in the red end. Actually, due to errors in counting large numbers of larvae often attached one on top of the other, the percentage was probably larger than this. In four of the trials there were 1.5 to 2 times as many in the red end. The standard error of the proportion was obtained from the formula, $S.E._p = \sqrt{pq/N}$, for calculating the significance of the results. If it is assumed that the null hypothesis holds in this case, the expected percentages in each end would be 50 per cent (.50), and both p and q would equal .5 each. Thus the $S.E._p = \sqrt{(.5 \times .5)/292} = .0291$. Since an excess of .17 (67 per cent minus 50 per cent) over that postulated by the null hypothesis is at least five times the $S.E._p$, the results would fall easily within the range of "very significant." In these experiments *B. flabellata* showed as much uniformity of response as *B. turrita,* despite the absence of eye-spots on the former. Visscher (1927) obtained somewhat similar results with colored tiles, for more Bryozoa attached to red test panels than to green, black or yellow ones, and no settings occurred on the white ones. (Cf. also Edmondson and Ingram, 1939.)

Darkness. As Grave (1930) had observed, darkness delays fixation and favors attachment to the surface of the water. The writer found the larvae of *B. turrita* to be almost universally active at the end of 24 hours, when kept in complete darkness in a microscope case, and the majority continued to swim for three or four days (the normal duration of the natatory period of most larvae of this species does not exceed 24 hours). Specimens kept in bottles remained active longer than those

in uncovered syracuse dishes. Since the sea water in the latter became more concentrated by evaporation, the increased salinity hastened metamorphosis. (Cf. Lynch, 1947). By six days the attached organisms had elongated considerably (maximum length, 1.45 mm.), giving the surface of the water a fuzzy appearance; this was caused by a great abundance of transparent material, much of it in the form of four, symmetrically placed stolons for attachment to the surface film and the remainder organized into a club-shaped structure joined to the stolons and containing in its center an opaque spherical mass that closely resembled the unmetamorphosed larva. The eye-spots were generally visible either in the opaque mass or at a short distance from it in the transparent parts. Apparently development ceased after elongation, for polypides were never observed.

Effects of temperature. Heating the medium to 30° C. accelerated metamorphosis, and raising the temperature to 32–35° C. caused cytolysis; both effects were more pronounced in *B. turrita* than in *B. flabellata.*[2] Many of the former, mere rings of ciliated tissue without material in their centers, were often observed swimming slowly in test tubes exposed to light from a 500 watt incandescent bulb. Enormous amounts of adhesive material always surrounded the larvae after extrusion of the hold fast, but rigid attachment failed to occur. Expansion, especially by elongation along the apico-basal axis, followed exposure to heat. Sausage-shaped streamers of tissue from the pallial furrow, similar to those produced by exposing the larvae to sea water containing an excess of magnesium chloride (Lynch, 1949), were extruded by the larvae of *B. flabellata* from their apical ends, which always looked larger than normal. Development was poor or totally lacking in both species, even when cytolysis did not occur. Marcus (1926) briefly mentioned the accelerating effect of heat on bryozoan larvae.

Geotropism. When diffuse daylight enters a test tube of sea water horizontally, the larvae become fixed at various places along the side farthest from the window, although attachment to the surface is also very common. Under experimenal conditions the larvae generally fell to the bottom just after immersion in a new medium, especially if it contained an excess of various salts. When sea water was mixed with equal parts of normal solutions of sodium, potassium, magnesium or calcium chlorides, the larvae did not swim to the surface again, apparently because ciliary action was too feeble. In mixtures of 80 cc. sea water per 20 cc. of normal calcium chloride, however, vigorous swimming movements were maintained. Heating the medium to 30° C., keeping the larvae in darkness or immersing them in 80 cc. sea water per 20 cc. normal sodium chloride favored attachment to the surface This concentration of sodium chloride, however, affected the two species somewhat differently. Since *B. flabellata* metamorphosed almost immediately in this mixture most of the larvae did not recover sufficiently from their initial "fright-reaction" to reach the surface, and floating larvae were not often observed; *B. turrita* re-

[2] Experiments on *B. neritina* had shown that a reduction of temperature from 23° C. to 7° C. caused all the larvae to become geopositive and lengthened the natatory phase by 2–3 hours (Lynch, 1947, p. 128). Since the writer was not interested in lengthening the larval phase of the Woods Hole species, similar experiments were not repeated. It cannot be assumed, however, that the results would have been similar, since Barrois (1879) reported that a bowl-full of Serialaria (Ctenostomata) that invariably attached at night, was placed in ice and maintained at a temperature near zero all night. Contrary to his expectations, setting took place in a normal manner.

mained active longer and many were at the surface when fixation took place. During the photonegative phase the larvae of *B. flabellata* swimming in normal sea water could be made to move downward by placing the light source above them or upward by illuminating them from below. (Cf. also Grave, 1930.) It seems likely, therefore, that in this species the positive geotropism that occurs in nature towards the end of the matatory period is brought about both by light and by a reduction in ciliary movement. In this respect the species at Woods Hole differ considerably from *B. neritina,* for geotropism in the latter is apparently independent of phototropic responses.

Discussion

The experiments just described and those presented in former papers (Lynch, 1947, 1949) show that heat, light, salinity and the relative proportions of ions in sea water can profoundly affect the natatory period of *Bugula* larvae and the subsequent growth of zooids. From an ecological standpoint, environmental fluctuations that affect the setting of larvae are of paramount importance. Hutchins (1945), having observed that adult species of Bryozoa grew quite well after being transplanted from their natural habitat to one where they were either rare or totally absent, concluded that "in all probability the critical tolerances of environmental variations are those of the larvae, particularly during metamorphosis when they may be supposed to be minimal." An interesting problem is posed by the peculiar distribution at Woods Hole of the two species of Bryozoa under discussion. Grave (1930) stated that *B. turrita* is found in Vineyard Sound, but not in the Eel Pond, whereas *B. flabellata* is abundant in the Eel Pond, but is not ordinarily found outside it, even though the two bodies of water are less than 300 feet apart and communicate freely with one another.[3] Although the adults when transferred from one region to the other on a raft may live for a few months or a year, all efforts to establish new colonies by the transplanted species have so far met with failure (Grave, 1930). A similar peculiar distribution of *Teredo navalis* and of certain hydroids can also be observed. What explanation can be given for these facts? That the larvae are extremely sensitive to the ions present in sea water is evident; whether they can complete metamorphosis and attain normal growth depends upon a very delicate balance of the chemical constituents of their environment. There are, however, so many variables affecting larval behavior that it is extremely difficult to isolate specific ones as causative agents of ecological distribution. Conceivably a particular species thrives best under conditions that enable the organism to terminate larval life after an optimum swimming-time, since larvae that are induced to swim long beyond the normal time of setting rarely develop or form zooids comparable to the controls in size or differentiation. Species such as *B. neritina* with an extremely short natatory period under laboratory conditions seem to be more adversely affected than those whose larval life is of longer duration. It may be that stored nutritive material essential for the formation of zooids is exhausted by prolonged swimming.

The behavior of the two species of *Bugula* from Woods Hole shows certain marked similarities to that of *B. neritina* (from the Beaufort region) and a few

[3] He referred, of course, to the natural habitat of the two species. Actually both species were found by the writer growing on a raft at the entrance to the Eel Pond (August, 1949).

striking contrasts. Their reactions to excesses of various metals are nearly identical (Lynch, 1947, 1949). (Copper, however, was not tried on *B. neritina.*) But there are significant differences in their responses to light and gravity. The larvae of *B. neritina* never have a photonegative phase at any time under laboratory conditions, although there is some evidence that they become indifferent to light just before setting. In this respect they resemble the European variety of *B. flabellata* described by Nitsche (1870). Furthermore, they almost universally remain near the surface, and up and down movements in a vial occur but rarely. The larvae of both species from Woods Hole, however, swim vertically along the side of the container during their photonegative phase as readily as they do horizontally during the transitional period when their phototropic responses are beginning to reverse.

The marked similarity in both phototropic and geotactic behavior of the two species from Woods Hole and the contrast that exists between the behavior of these species and that of *B. neritina* would suggest the possibility that environmental conditions in the two regions might be partially responsible for differences in behavior. Both the extreme brevity of the natatory phase of the Beaufort species and the failure of the larvae to become geopositive at any time under laboratory conditions would seem to be influenced at first sight by two obvious differences in environment, a higher temperature and a brackish condition of the water. This hypothesis is based on the experimental evidence that extreme variability of the duration of the natatory period of a given species can be brought about by altering the ionic balance of the medium, by changing the salinity or by varying the temperature. By altering these factors the geotropic behavior of the larvae can also be changed. Since the abundance of oysters in the brackish waters of Beaufort would suggest the probability that the copper content of this region might be greater than it is at Woods Hole, it would not be unlikely that this ion, capable of hastening the onset of metamorphosis in several sessile organisms, might be largely responsible for the extreme brevity of the larval stage of *B. neritina.*[1] The hypothesis that a greater concentration of copper in the Beaufort sea water and the higher temperature prevailing in that region might play a role in causing contrasts in behavior of the northern and southern species is not illogical, since the natant phase of *B. flabellata* can be shortened appreciably either by adding $CuCl_2$ to sea water or by raising the temperature of the medium. Furthermore, warming the sea water to 30° C. favors surface attachment of *B. flabellata,* whereas cooling the medium causes the larvae of *B. neritina* to become geopositive and prolongs their larval stage; thus, either species may be made to react like the other in this respect. Apparently the pelagic habits of *B.*

[4] In brackish waters, according to Prytherch (1934), the copper content may reach a concentration of 0.1–0.6 mg. per liter during low tide, whereas it rarely exceeds 0.02 mg./liter in the sea (Galstoff, 1943). Likewise the relative proportions of sodium, magnesium and calcium in brackish waters differ considerably from the distribution of these ions in the ocean, since the order of concentration of these ions approaches that of fresh water (calcium, magnesium and sodium). (Cf. Clarke, 1924.) Recently (after this paper had been prepared in its present form) Glaser and Anslow (1949) gave the copper content of Woods Hole sea water (spectroscopically determined) as $2.50 \pm \times 10^{-7}$ M Cu. They found that a sample of Beaufort sea water had a value as high as 1×10^{-6} M Cu; they noted, however, that the latter may have been contaminated (p. 127 and 128).

neritina are correlated with their brief natatory period (Lynch, 1947);[5] likewise the much more frequent occurrence of geopositive settings of *B. flabellata* under normal conditions seems to be related to their naturally longer larval phase, for the number on the bottom of a vessel begins to increase after the larvae have been active for several hours.

Nevertheless, even though the behavior (except phototropism) of one species can be duplicated almost exactly in the other by altering the environment, neither differences in temperature *alone* nor in the content of the sea water can account for contrasts in the behavior of the northern and southern species. The monthly mean temperatures during July and August at Woods Hole and at Beaufort differ by only six or seven degrees, according to McDougall (1943), and experiments performed at Beaufort by the writer showed no marked change in the behavior of *B. neritina* when the temperature was reduced to 21° C. (the monthly mean for Woods Hole), although a more drastic reduction of temperature did reverse the geotropism of the larvae and prolong their free-swimming phase. Likewise, when larvae of *B. flabellata* were immersed in sea water taken at low tide from the Beaufort region and shipped to Woods Hole, their behavior was like that of the controls. No shortening of the larval phase was observed. (The experimental sea water had a pH of 7.5 when used.) Logically, negative results were partially predictable, since previous experiments had shown that a concentration of copper chloride as low as 0.5 mg per liter of sea water (about maximum for brackish waters) had no appreciable effect in shortening the natatory period, although higher concentrations were effective.[6] Is it then a mere coincidence that the southern species with its short natant phase lives in an environment where two factors, a higher temperature and a presumably (?) greater concentration of copper, are both present and either of these can accelerate the onset of metamorphosis? There are two possibilities. On the one hand, since experimental modifications of temperature that proved to be effective in lengthening or shortening the larval phase were more drastic than those actually prevailing in nature and since the same was true of the copper content, conceivably slighter changes of both factors combined might be as effective as more extreme alterations of each one separately. It should be noted that, since the temperature of the Beaufort sea water was not raised to the degree ordinarily prevailing in that region, the environment of the southern species was only partially duplicated on the northern one. On the other hand, since there are generic and specific as well as individual differences in the natatory phase of bryozoan larvae, it seems more tenable to assume that species genetically determined to have a short larval period can thrive only in an environment where ions, presumably requisite for setting, can be rapidly absorbed. Conceivably, there may be specific differences in the ion-absorbing ability of larvae. This assumption, however, offers no explanation for the fact that the larval phase of the majority of a given species may end at two hours on one day and at ten hours on another.

[5] By using two sets of data that appeared to have the least positive correlation, the coefficient of correlation between the number of larvae of *B. neritina* that were kept active for four hours by a reduction of salinity and the number that became geopositive at the time of setting was found to be +.53 and +.57.

[6] It should be noted that concentrations of $CuCl_2$ as high as 1.25 mg. per liter had no effect; Prytherch (1934) found that in his experiments virtually all the copper was precipitated when less than .5 mg./liter was used.

(See Table I, p. 30, Lynch, 1949).[7] Before the relative effects of environment can be evaluated it is necessary to know whether there are significant differences in the length of the natatory period of the same species in different localities. Since Edmondson and Ingram (1939) reported that larvae of the Hawaiian *B. neritina* attach at night as readily as during the day, it may be that these organisms have a much longer natant phase than the ones studied at Beaufort. The latter (laboratory conditions) were always released a short time after exposure of the parental colonies to light, and active unattached ones could rarely, if ever, be found after noon. Caution must be observed, however, in making such an assumption, for it is unwise to conclude that the natatory period of larvae under natural conditions is as brief as it is in the laboratory. Indeed, the vertical distribution of adults indicates that in nature these larvae are probably active longer than an hour or two (maximum time in the laboratory) and undergo sufficient activity to make most of them geopositive. It would be extremely valuable to have definite information regarding the behavior of *B. neritina* on the California coast. There is also need for further research on the interaction of factors capable of accelerating or retarding metamorphosis. Some may have antagonistic effects; others may act synergistically.

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Summary

1. The larvae of both *B. flabellata* and *B. turrita* are photopositive in diffuse light during the first 3–4 hours after release from the ovicells and then become photonegative. They became photonegative immediately, however, when they were placed in sea water buffered to a pH of 9.6 or diluted by 50 per cent; sea water containing copper chloride either reduced the intensity of the photopositive phase or caused a reversal of phototropism depending on the concentrations that were used. These organisms became indifferent to light in mixtures of 80 cc. sea water per 20 cc. of either normal calcium chloride or magnesium chloride.

2. Larger numbers of larvae attached in the red end of a test tube illuminated by red and blue light passing through Wratten filters than in the opposite end.

3. Heating sea water to 30° C. hastened metamorphosis and favored surface attachment, but development was poor or entirely lacking at this temperature. Darkness delayed metamorphosis and also caused attachment to the surface film;

[7] Barrois (1879) stated that in the laboratories at Roscoff the same species of Bryozoa generally showed extreme variations of the natatory period on different days and that cases of this strange phenomenon could be found in all groups but was especially striking in *Pedicellina* and *Cyphonautes*. In some cases (*Flustrella hispida*, especially) he found it impossible to obtain a single fixation during a period of many weeks, even though the larvae were very abundant. At other times, under apparently identical conditions, fixations took place in large numbers. He noted that the incapacity of larvae to fix themselves might persist for a long time or cease suddenly. At times settings occurred in various parts of the bowls; at other times they took place *en masse* at certain points. But these anomalies were entirely absent in bowls prepared at the same time and place and under identical conditions Harmer (1922, p. 513), likewise, noted the difficulty of persuading larvae to attach under laboratory conditions and stated that it could be surmounted by placing adult colonies in a vessel closed with fine muslin and left attached to a buoy or placed in a deep tide-pool. Evidently environment is extremely important.

development ceased after a fair amount of growth and a slight degree of differentiation.

4. In mixtures of equal amounts of sea water and normal solutions of sodium, potassium, magnesium and calcium chlorides the larvae became geopositive on entering the medium and remained so during the experiments.

5. Some ecological problems of the distribution of three species of *Bugula* are discussed and tentative suggestions for their solution are offered.

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THE RESISTANCE OF SCIARA (DIPTERA) TO THE MUTAGENIC EFFECTS OF IRRADIATION

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On the basis of the irradiation studies made on Sciara to date, a condition has been noted which is of general interest with reference to the mode of action of x-rays on the hereditary material, namely, an apparent resistance to the mutagenic effects of irradiation. Whereas gross and minute chromosome rearrangements are induced in treated germ cells, visible mutations appear at a negligible frequency.

Sciara is very unusual in this respect. In other organisms, including Drosophila, maize, and Neurospora, x-rays are found to induce both mutations and chromosome breaks at frequencies proportional to the dosage. During the past twenty years at least eight investigators have independently looked for visible mutations among the progeny of irradiated Sciara; altogether only twenty-four or twenty-six mutant characters have been obtained in the several species treated. Unfortunately, the irradiation data are not tabulated in such a way that an estimate can be made of the number of germ cells exposed or the total progeny examined. But certain of the studies were extensive. Both male and female germ cells were treated at various times in the developmental cycle and dosages from 3000 to 30,000 r. applied.

In view of the low mutation rate obtained repeatedly (see Metz, 1938), the chromosomes of Sciara were believed to be resistant to irradiation. The first clue to the contrary was the discovery of a reciprocal translocation in the salivary gland nuclei of larvae taken from cultures of the "Stop" mutant (Crouse and Smith-Stocking, 1938). The salivary gland chromosomes were then utilized in a cytological analysis of F_1 larvae derived from irradiated sperm or oocytes (Metz and Boche, 1939); following exposure of sperm to 5000 r., approximately 25 per cent of the F_1 showed gross chromosomal rearrangements. This unexpected induction of chromosome aberrations in Sciara was confirmed in subsequent experiments.

The experiments reported in this paper were performed in connection with cyto-genetic studies on the unusual behavior of the sex chromosome of Sciara. The data bear on the problem at hand, however, and will therefore be discussed in this relation.

Preliminary data on dominant lethal induction are in line with the rearrangement studies and provide further evidence that the chromosomes of Sciara are sensitive to irradiation. In one experiment designed to pick up X-translocations in *S. coprophila,* nine females were bred singly to adult males which had been x-rayed at 4000 r. The nine females yielded 108 total offspring, while nine control females from the same stock (isogenic) produced 723 total offspring. The exact probability (as measured by chi-square) of this difference is 0.0004. On the basis

of this small but very carefully conducted experiment, only 15 per cent emergence was obtained, a dominant lethal value which is practically identical to that measured by Demerec and Fano (1944) in Drosophila sperm x-rayed at 4000 r. It is of interest to note that in the Sciara experiment cited, nine of the 108 survivors (8 per cent) were heterozygous for X-translocations but none transmitted sex-linked visible mutations.

If dominant lethals are regarded as the result of certain types of chromosomal aberrations (see Pontecorvo, 1942), Sciara and Drosophila chromosomes respond to irradiation in a similar manner, and the physiological result (i.e., the lethal phenotype) is the same in both genera. It is with respect to less drastic physiological changes—namely, hereditary alterations classified as "visibles"—that the two genera differ.

Several factors may account for the low visible mutation rate in Sciara.

(1) The external appearance of this fly (bristle pattern, pigmentation, etc.) is such that only the most conspicuous visible changes are likely to be detected.

(2) There is a distinct difference between the autosomal and the sex-linked mutations, which suggests that the induced mutation rate in this genus is considerably greater than the detected mutation rate. In *S. coprophila,* the species which has been most thoroughly worked, nine autosomal and five sex-linked mutations have been recovered. Of the autosomal group, seven are dominant and two are recessive; among the sex-linked factors, on the other hand, four are recessive and only one is dominant. The exact probability (as measured by chi-square) of this difference is 0.126. In material such as Drosophila, Habrobracon and maize, the dominant mutations constitute a very small percentage of the total number of visible mutations. The relatively high proportion of dominants in Sciara has been interpreted as evidence that this genus is unique in its response to irradiation (Metz, 1938). Such an interpretation is probably not valid, since, as noted above, the dominants constitute a majority only in the case of the autosomal factors. Most likely the discrepancy in the autosomal mutations has a relatively simple explanation, namely the extraordinarily tedious and inefficient technique available for the detection of autosomal recessives in this genus.

The mode of inheritance and the mechanism of sex determination in Sciara make it difficult to pick up autosomal recessive mutations *S. coprophila* is monogenic This means that the females produce either sons or daughters but not both; consequently, an F_1 female derived from an irradiated sperm will yield a family of sons or a family of daughters. If she is a male-producer and heterozygous for an induced sex-linked recessive, half of her sons should show the mutant character. Sex-linked recessives, therefore, can be fairly readily detected in Sciara in the F_2 generation. Autosomal recessives, on the other hand, are practically impossible to pick up because of the monogenic condition described above and because Sciara males transmit only the genes they inherit from their mother; the paternally derived chromosomes are eliminated at the first spermatocyte division. In searching for autosomal recessives, the best procedure is to take the mutated gene (a mutation induced in treated sperm), through the female germ line according to the scheme outlined below.

In order to detect the recessive mutation, a, four successive generations of flies (approximately four months at 70° F.) have to be produced as outlined; and then

P_1 ♀-producing ♀ by treated ♂

F_1 Aa ♀-producing ♀ by AA ♂

F_2 Aa ♀-producing & ♂-producing ♀♀ by AA ♂♂

F_3 Aa ♀♀ Aa ♂♂

F_4 1 Aa : 1 aa

the probability that the mutation will be detected in the F_4 is less than $\frac{1}{16}$. The calculation, of course, does not include I, the probability of induction of the mutation. In the sex-linked mutant strains of *S. coprophila,* male- and female-producers are phenotypically distinguishable. If one of these mutant strains is used in the experiment diagrammed above, the chance of selecting a ♀-producing female and a ♂-producing male of genotype Aa in the F_2 is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$ (F_2 females are AA or Aa). Then the chance of selecting a female and a male of genotype Aa in the F_3 is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$, making the final probability $I \times \frac{1}{4} \times \frac{1}{4}$. Thus, the odds against detection of an autosomal recessive in *S. coprophila* are at least 15:1.

In digenic species like *S. reynoldsi* (the females produce both sons and daughters), autosomal recessives can be detected in the F_3 generation according to the following scheme:

P_1 ♀ by treated ♂

F_1 Aa ♀ by AA ♂

F_2 Aa ♀♀ and Aa ♂♂

F_3 1 AA : 1 aa

In this case the chance of selecting an F_2 male and female of genotype Aa is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$, making the odds against detection of the mutation only 3:1 instead of 15:1.

The evidence presented so far is consistent with the view that the detected mutation rate in Sciara is considerably less than the induced rate. Even so, the induced rate appears to be less than that observed in Drosophila or Habrobracon; for, in the case of the sex chromosome of *S. coprophila,* where recessives can be picked up in the F_2, only five factors (four recessive and one dominant) have been found.

This dearth of mutants is particularly significant in view of the fact that over a period of several years the author has tested a large number of irradiated sperm (approximately 500) and oocytes (approximately 100) of *S. coprophila* in a search for reciprocal translocations which involve the X chromosome (Crouse, 1943 and

unpublished). In these experiments, breeding tests were carried to the F_2 generation or beyond; fourteen analyzable (genetic and cytological) X-translocations have been recovered but not a single visible sex-linked or autosomal mutation. Similar experiments conducted on *S. reynoldsi* yielded four X-translocations and no mutations among irradiated sperm (approximately 200 sperm tested).

(3) The presence in the germ cells of *S. coprophila* of large amounts of heterochromatin, in the form of the limited chromosomes, may actually retard the mutation rate. There are no published data on the effects of heterochromatin on general mutability either in corn or Drosophila—two forms in which the B-type chromosomes and the Y, respectively, might well be exploited. Unpublished studies of Miss Jean Kerschner, on the effects of an extra Y chromosome on the x-ray induced visible and sex-linked recessive lethal rates in *D. melanogaster* males, indicate that the extra Y brings about a significant decrease in these mutation rates.

(4) Finally, it is conceivable that, as compared to Drosophila or Habrobracon, the biochemical pathways which are available in Sciara result in a more restricted range of phenotypic variability.

Summary

Several facts have been discussed which may contribute to the apparent resistance of Sciara to the mutagenic effects of irradiation. New data are presented which (1) support the view that the visible mutation rate induced in this genus is lower than that found in Drosophila or Habrobracon; and (2) reveal a high dominant lethal mutation rate in this genus.

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RECOVERY FROM ULTRA-VIOLET LIGHT-INDUCED DELAY IN CLEAVAGE OF ARBACIA EGGS BY IRRADIATION WITH VISIBLE LIGHT [1]

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Kelner has reported that exposure of *Streptomyces griseus* conidia to intense visible light (V.) after inactivation with ultra-violet light (U.V.) enabled these conidia to grow (Kelner, 1949). Dulbecco obtained similar results when bacteria containing U.V.-inactivated bacteriophage were given visible light (Dulbecco, 1949). The experiments to be described show that a similar phenomenon occurs when *Arbacia punctulata* eggs fertilized with U.V.-treated sperm are exposed to intense visible light.

Methods

The U.V. source was a Westinghouse Sterilamp W.L. 782, 80 per cent of whose radiation was at $\lambda = 253.7$ mμ. The visible light was produced by two 1000-watt tungsten filament lamps, the light from each being focused through a round, water-filled, one-liter pyrex flask onto a white tile. An infra-red filter was placed 5 inches from the illuminated spot which was 2 inches in diameter. A fine-grained ground-glass screen was placed 2 inches above the tile in order to diffuse the filament images. The U.V. intensity, measured with a Hanovia Ultraviolet meter, was 52 micro-watts per cm^2 at the position where the biological material was irradiated. The visible light intensity on the white tile, as measured with a Leeds-Northrop Macbeth Illuminometer, was approximately 28,000 foot candles. The rise in temperature of the irradiated suspension, as measured with a thermometer immediately after irradiation, was less than 0.2° C. The heat generated by the lamps was dissipated by circulation of air with an electric fan. With windows and doors of the laboratory kept open, there was no appreciable rise in the temperature of the room.

Sperm suspensions were prepared by dispersing 2 drops of "dry sperm" in 250 ml of sea water ("0.4 per cent sperm"). Egg suspensions contained 5–10,000 eggs/ml. For irradiation with either V. or U.V., 2 ml of either suspension was pipetted into a pyrex petri dish (without cover) 2 inches in diameter, which formed a layer about 1 mm high. To fertilize eggs, 0.2 ml of the sperm suspension was added to the 2 ml egg suspension with agitation.

The time for cleavage was obtained by counting the number of cleaved and uncleaved eggs, the total being 50 to 100 eggs each time. After determining the point at which 50 per cent of the eggs cleaved, they were again counted to determine total cleavage. Data were discarded unless the controls showed a final cleavage of 95 to 100 per cent.

[1] Supported by a grant from the U. S. Public Health Service.

Temperatures were recorded but not controlled. In all experiments, temperature fluctuations were less than 1° C. in the course of the experiment.

Results

1. *Time for cleavage as a function of U.V. dose*

Sperm were irradiated for periods from 0.5 to 4.0 minutes and unirradiated eggs fertilized 8 to 10 minutes later. Since at the higher doses cleavage did not reach 50 per cent, comparison was made at the 10 per cent cleavage point. The following table gives the results obtained:

(24° C.)

Exposure time (min.)	0	0.5	1.0	2.0	3.0	4.0
Time to 10 per cent cleavage (min.)	47	88	113	157	214	235

Eggs were treated with U.V. and then fertilized by normal sperm with results as shown below:

(24° C.)

Exposure time (min.)	0	0.5	1.0	2.0	3.0	4.0
Time to 50 per cent cleavage (min.)	47	49	58	62	68	72

2. *Growth of the U.V. effect*

Sperm and egg suspensions were irradiated with U.V. and allowed to stand for varying lengths of time before fertilization. The sperm was irradiated for 0.5 minutes, the eggs for 5.0 minutes. The following results were obtained when irradiated sperm was used to fertilize normal eggs and irradiated eggs were fertilized with normal sperm:

Time for 50 per cent cleavage (25° C.)

T	2	10	20	30	40	50	60	80	c	c′
Irradiated sperm	98	100	98	104	107	110	120	124	44	47
Irradiated eggs	69	71	69	68	68	68	73	70	47	47

T = time in minutes from irradiation to fertilization
c = non-irradiated eggs and sperm. Fertilization immediately after preparation of suspensions.
c′ = non-irradiated eggs and sperm. Fertilization 75 minutes after preparation of suspensions.

There was an appreciable growth of the cleavage delay factors in the irradiated sperm, but there was neither growth nor decay of these factors in the egg.

3. *Irradiation with ultraviolet and visible light before fertilization*

To determine whether V. given to either gamete before or after U.V. irradiation altered the U.V.-induced cleavage delay, eggs and sperm were separately irradiated. The irradiated sperm were used to fertilize normal eggs and irradiated eggs fertilized with normal sperm. The results are given below:

Time for 50 per cent cleavage (25° C.)

	Exposure time V.	Exposure time U.V.	Control	U.V.	V.	V. then U.V.	U.V. then V.
Sperm irradiated	3′	10″	46	70	46	69	68
	3′	30″	46	117	—	114	109
	3′	2′[2]	41	139	—	135	133
Eggs irradiated	3′	2′	48	72	47	72	74

Visible light given to either gamete did not change the cleavage time. Visible light given before or after U.V. did not significantly shorten or prolong the U.V.-induced delay in cleavage.

4. *Visible light given to the zygote*

When visible light (3 minute exposure) was given to eggs fertilized with untreated sperm at various times after fertilization, the time for cleavage was not significantly affected. The following shows the results obtained:

V. – 3′ t – 27° C.

I	2	5	10	15	20	30	c
T	41	41	40	43	42	44	44

I = Time of irradiation with visible light in minutes after fertilization. I is measured to the time of beginning of irradiation.
T = Time in minutes for 50 per cent cleavage.
c = Control not exposed to light.

However, when the zygote was exposed to visible light after fertilization with U.V.-treated sperm, there was marked reduction in the time for cleavage. The following are results from three runs at different times to indicate the degree of reproducibility. In all cases the eggs were fertilized within 10 minutes after U.V. irradiation of the sperm. Repetition of these experiments several times gave essentially the same results:

U.V. – 0 5′, V. – 3′

I (26° C.)	1.5	5	9	11	15	20	30	40	60	c′	c
T	60	58	56	54	69	81	91	94	98	97	44
(27° C.)	2	6	10	35	50					c′	c
T	65	60	56	85	90					97	43
I (25° C.)			10	50	60	70	80			c′	c
T			59	87	90	93	95			96	44

I, T, and c as above.
c′ = eggs fertilized with U.V. treated sperm but not exposed to visible light.

The delay in cleavage (inactivation) is considerably shortened when exposure to V. begins as early as 1.5 minutes after fertilization. This shortening of the cleavage-delay time will be referred to as photoreactivation. Maximum efficiency in photoreactivation was observed when exposure to visible light began at 9 to 11 minutes after fertilization. Detectible photoreactivation was observed as late as 40 to 50 minutes after fertilization.

[2] In this series, values are for time to 5 per cent cleavage.

Neither U.V. inactivation nor photoreactivation had an appreciable effect on the time for the second cleavage. Below are given the observed times between first and second cleavages after U.V.–0.5′ and V.–3′ at the times indicated:

I	10	50	60	70	80	c′	c
T[3]	26	30	32	32	30	32	26

I, T, c′, c as above.

It was noted in some experiments that c′, and the samples receiving V. at times later than 20 minutes after fertilization, had a considerable percentage of the eggs with a 3 to 4-cell first cleavage. This occurred at about the same time that the remaining cells showed the usual 2-cell cleavage. These 3 to 4-cell first cleavages were absent or rare in c and in eggs receiving V. at 10 to 20 minutes after fertilization. In these cases when allowance was made for the percentage of 3 to 4-cell zygotes by first cleavage, and the time determined for 2-cell zygotes to reach the 4-cell stage, the results were essentially the same as those given above. The factors responsible for the production of 3 to 4-cell first cleavages were not investigated.

5. *Quantity of light for maximum photoreactivation*

Following irradiation of sperm with U.V. for 0.5′, eggs were fertilized and the fertilized eggs exposed to the visible light, exposure beginning at 10′ after fertilization in all cases. The exposure time was varied with the results shown below:

$t - 24°$ C.

E	10	30	60	120	180	300	c′	c
T	87	77	75	78	63	66	99	58

E = exposure time in seconds.
T, c′, c as above.

The data show that exposure for periods longer than 3 minutes did not produce greater photoreactivation.

6. *Photoreactivation in nucleated white halves of Arbacia eggs*

Eggs were centrifuged at approximately 10,000g for 10 minutes and the nucleated white halves and non-nucleated red halves collected separately. These were then fertilized with U.V. treated sperm and the zygotes exposed to visible radiation with the following results for the white halves:

U.V. – 0.5′, V. – 3′ $t - 26°$ C.

I	10	20	30	50	65	c′	c
T	72	83	95	100	101	106	46

I, T, c′, c as above.

Photoreactivation was, therefore, possible in the non-pigmented half-eggs although the capacity for photoreactivation appears to be lower than in the whole egg. Since cleavage in the red halves was rare, the presence or absence of the capacity of these pigmented half-eggs for photoreactivation was not investigated.

[3] Time measured from 50 per cent first cleavage to 50 per cent second cleavage.

7. *Photoreactivation efficiency of light of different wave-lengths*

Wratten filters, 3″ × 3″, were mounted on the ground glass screen with the edges held by scotch tape, and oriented so that all of the beam passed through the filters. With filter No. 73, which transmitted at 560 to 620 mμ (maximum 570 mμ) and to some extent at 680 to 700 mμ and had a total transmission of 1.6 per cent, the following results were obtained:

		U V. – 0 5′, V. – 8 0′			t – 24° C.	
I	10	20	60	c′	c	c″
T	80	94	107	106	50	57

I, T, c′, c as above.
c″ = full visible spectrum for 3′ at 10′ after fertilization.

Filter No. 76, which transmitted at 330 to 470 mμ (maximum at 440 mμ) and very little at 690 to 700 mμ with total transmission at all wavelengths of 0.1 per cent, gave the following results:

		U V. – 0 5′, V. – 8 0′			t – 24° C.	
I	10	20	60	c′	c	c″
T	80	94	126	120	51	57

I, T, c′, c, c″ as above.

The same amount of photoreactivation was obtained with either filter. Since the total transmission of No. 76 was less than that of No. 73 by a factor of 16, the photoreactivation efficency of the band 330 to 470 mμ was much greater than light of wavelengths 560 to 620 mμ. The intensity of the filtered light was too low to obtain maximum photoreactivation in both cases, but this does not affect the validity of the above deduction.

8. *Time for the "streak" stage*

In addition to the time for 50 per cent cleavage, the time when the "streak" stage first appeared was also noted. In 6 experiments, this time varied from 13 to 18′, but in each case the time was the same, within one minute, for the non-irradiated controls and those which had received U.V.-treated sperm. Since the streak represents the development of the sperm aster, U.V.-irradiation apparently affects a later stage.

9. *Treatment with chemical agents* [4]

Streptomycin, in concentrations of 1 μg/ml to 10 μg/ml, had no effect on cleavage time of eggs fertilized with normal sperm. When sperm were given U.V. in the presence of streptomycin (1 mg/ml), there was no significant change in either U.V.-inactivation or photoreactivation.

Treatment of eggs with adenosine (20 μg/ml), prior to fertilization with U.V.-treated sperm, did not alter cleavage time, nor did it affect photoreactivation. Ad-

[4] We are indebted to the Lederle Laboratories for the folic acid and 4-amino-n-methyl folic acid, the Merck Laboratories for the streptomycin, and the Wellcome Research Laboratory for the 2,4-diamino-5-p chlorophenoxypyrimidine.

dition of adenosine to sperm immediately after U.V.-irradiation and fertilization 20′ later, also had no effect.

Eggs treated with folic acid at concentrations up to 10 μg/ml for 20′ before fertilization, or at 7′ after fertilization, with U.V.-irradiated sperm, showed no change in cleavage-delay time.

Eggs treated with 4-amino-n-methyl folic acid (10 μg/ml), 5′ after fertilization and for 20′ before fertilization, with U.V.-treated sperm, showed no effect on cleavage delay or photoreactivation.

Eggs were also treated with the purine antagonist 2-4-diamino-5-p-chlorophenoxypyrimidine (Falco and Hitchings, '49) for 30′ before fertilization with normal and with U.V. treated sperm. No effect was observed on the normal cleavage time, the U.V. inactivation or photoreactivation.

When U.V.-irradiated sperm were treated with riboflavin (20 μg/ml) or with riboflavin (20 μg/ml) plus adenosine (20 μg/ml) for 15′ before their use in fertilization, there was no effect on cleavage delay. Neither of these substances nor both given together affected normal cleavage time. When sperm treated with riboflavin were exposed to the visible light, they showed first a peculiar rapid vibratory motion and then complete cessation of motion within 10 minutes. Eggs could be fertilized with such sperm immediately after exposure to light. When eggs were thus fertilized, the time for cleavage was prolonged much beyond the usual U.V.-delay time. The cells attempting to cleave became very much elongated and twisted, but were only partially constricted. However, eggs treated with riboflavin for 20′ before fertilization with U.V.-irradiated sperm and not exposed to visible light, cleaved normally and showed no change in the cleavage delay time.

Discussion

Kelner (1949) and Dulbecco (1949) have both suggested that photoreactivation may be a general biological phenomenon. The experiments described here indicate its presence in an organism very different from either of those previously studied. It is interesting that in this respect the combination bacterium-phage is analogous to the combination egg-sperm. This analogy suggests that it would be desirable to determine whether inactivation of the phage could be obtained by U.V. irradiation of the bacterium prior to infection, and whether photoreactivation then follows the same course as in the case of U.V.-irradiated phage.

The present experiments show that there is a critical stage in the interaction of the sperm and egg when susceptibility to photoreactivation is at a maximum. The experiments on the time for the second cleavage also show that the cleavage-delaying conditions or agents are removed by the end of the first cleavage. The delay in cleavage is not due to inhibition or delay in the formation of the sperm aster. The effect must therefore be on some later stage. Once the cleavage constriction begins, the time for completion of cleavage is not significantly different in eggs receiving normal or U.V.-treated sperm within the dosage limits used in these experiments. The overall delay may therefore be attributed to a delay in the onset of prophase or to a prolongation of the prophase-metaphase time, or both. Since maximum sensitivity is at the time of onset of prophase, this appears to be the stage at which the U.V. inhibition becomes effective.

The agent or condition produced by U.V. remains unaltered in the unfertilized

egg but increases with time in the sperm, suggesting that stabilizing factors present in the egg are absent in the sperm, so that reactions initiated by U.V. absorption are much retarded or stopped in the egg but not in the sperm. The Arbacia egg contains a very considerable amount of ribonucleic acid in its cytoplasm (Schmidt, Hecht and Thannhauser, 1948 and Brachet, 1933). Consequently the relative inefficiency of U.V. irradiation of the egg may be taken as an indication that U.V.-alteration of ribonucleic acid of the cytoplasm is not involved and that the system affected is in the nucleus. The occurrence of photoreactivation in the non-pigmented half-egg indicates that the pigment is not involved in this reaction; and since most of the granules are removed, the latter also are probably not involved.

The relatively high efficiency of light of wavelengths in the region 360 to 460 mμ, suggests the possibility that intracellular substances with absorption bands in this region may somehow be related to the photoreactivation phenomenon. Riboflavin and folic acid both have absorption bands in this region (Warburg and Christian, 1938 and Stokstad, Hutchings and Subbarow, 1946). However, folic acid given to eggs or sperm does not alter the U.V.-inactivation. Also the folic acid inhibitor, 4-amino-n-methyl folic acid, does not prolong cleavage of the normally fertilized egg nor alter the U.V.-induced cleavage delay. The purine antagonist, 2-4-diamino-5-p chlorophenoxypyrimidine, also has no effect. No definite conclusion can be drawn from the failure to observe effects with any of these substances, since there is no proof that they penetrated the eggs or sperm. An exception may be made in the case of riboflavin since a marked effect was observed when the eggs or sperm were exposed to light. However, the effects observed with riboflavin-treated irradiated sperm seem to be unrelated to photoreactivation. Eggs treated with riboflavin, and U.V.-irradiated sperm similarly treated, show the usual cleavage delay time. The prolongation of cleavage, and abnormalities thereof produced by sperm irradiated with visible light in the presence of riboflavin, may be attributed to toxic photodecomposition products of riboflavin.

Conclusions

1. Irradiation of either sperm or egg with ultraviolet light will delay the time for the first cleavage division of the zygote formed by such gametes. Since irradiation of the egg is much less effective, the U.V.-inactivation is probably produced in the nucleus. The time of the formation of the sperm aster is not affected.

2. There is no decay or growth of the U.V. effect with time, after irradiation of the egg; but in the sperm there is definite growth.

3. Irradiation of either gamete with intense visible light, after U.V. irradiation, does not alter the cleavage delay.

4. Irradiation of the zygote with intense visible light reduces the cleavage-delay time. The zygote is most reactive at 10 minutes after fertilization, which is approximately the time of onset of prophase, although detectible response may be observed as late as 50 minutes after irradiation. The mitotic stage which is delayed and which responds most to the visible light is the onset of prophase.

5. Treatment with adenosine, streptomycin, folic acid, and 2-4-diamino-5-p chlorphenoxypyrimidine had no effect on either U.V.-inactivation or photoreactivation.

6. Treatment of the egg or zygote receiving U.V.-inactivated sperm with riboflavin, had no effect on the cleavage time. Irradiation of sperm in the presence of riboflavin with visible light, had a toxic effect on the sperm and on the zygote formed from such irradiated sperm.

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TRACHEAL FILLING IN SCIARA LARVAE

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INTRODUCTION

During insect development the gas-filled tracheae of a given instar become enclosed in larger, liquid-filled, coaxial tubes which are to form the tracheal system of the next instar. At molting the old tracheae are withdrawn and shed with the body cuticle. The new system then fills with gas, either immediately or within a short time, the liquid probably passing through the tracheal wall into the blood or tissues (Weismann, 1863; Palmén, 1877; Keilin, 1924, 1944; Davies, 1927; Wigglesworth, 1938; Keister, 1947, 1948). We shall call the process by which gas replaces the tracheal liquid "tracheal filling."

It is frequently assumed that the gas which appears in the new tracheae of insects with open tracheal systems is atmospheric air which has entered through the spiracles. This assumption is supported by the fact that in some insects the tracheae do not fill with gas unless the spiracles are exposed to free air. In other species, however, the tracheae can fill with gas even when the larva or embryo is submerged in water or surrounded by amniotic fluid, and many aquatic insects with closed systems normally fill their tracheae without apparent contact with an external source of gaseous gas (for literature see Keilin, 1924, and Sikes and Wigglesworth, 1931). It thus appears that in different insects there are at least two distinct mechanisms of tracheal filling, differing as to the source of the gas.

A number of explanations of tracheal filling have been proposed.

Weismann claimed that growth of tracheae (increase in diameter and number) continues after a change in permeability of the lining prevents entry of further liquid. The original liquid then retreats into the finer branches where it is absorbed, and gas enters from the outside or diffuses in from the tissues to occupy the increased tracheal volume. Major objections to this idea are the facts that, at least in Sciara, the tracheae have the same diameter when originally laid down as when they fill, and that no new branches are added near the time of filling (Keister, 1948).

Stadtman-Averfeld (1923) suggested that the violent body movements at molting expel the liquid from the tracheae, after which air enters through the spiracles.

Tillyard (1916), from the equivocal observation that tracheae collapsed when dragonfly larvae were put in KOH, proposed that the gas in the tracheae was CO_2 which diffused in from the hemolymph and displaced the liquid. Keilin (1924) pointed out several difficulties with this hypothesis. An additional objection is that the hydrostatic pressure in the tracheal liquid would have to be less than in the body liquids in order for gas evolution to be confined to the tracheae.

Filling has been attributed to reduction in body hydrostatic pressure at hatching (Davies) or after eclosion (Fraenkel, 1935). However, such reduction would not bring about the disappearance of the tracheal liquid unless the pressure had pre-

viously opposed some other force (e.g., osmotic) tending to withdraw the liquid into the body. Furthermore, it would not necessarily lead to the concurrent appearance of gas in the tracheae except in insects with spiracles open to the air. In closed tracheal systems, reduction in hydrostatic pressure might indeed bring about a preferential evolution of gas in the tracheal liquid (in the unlikely event that the tracheal liquid was higher in dissolved gases than the other body liquids) but would not account for the disappearance of the liquid itself. In any case, the facts that some embryos fill their tracheal systems before hatching, and that many insect eggs apparently have a low internal pressure (Sikes and Wigglesworth), argue against the idea that hydrostatic pressure has any major role in tracheal filling.

From ingenious experiments on the osmotic control of gas movement in the "tracheoles" of mosquito larvae, Wigglesworth (1930) postulated that increased tissue osmotic pressure due to muscular activity at molting could explain tracheal filling. Such a mechanism might conceivably lead to the replacement of tracheal liquid by outside air in insects with open systems, if the tracheal wall were semipermeable and the tracheal liquid markedly hypotonic to the body liquids as Wigglesworth postulated. However, neither of these conditions has been shown directly and unequivocally to occur, and indeed the latter seems rather improbable in view of Keilin's (1924) and Wigglesworth's (1939) opinion that the tracheal liquid is molting fluid, and Keister's demonstration that it originates by direct cytoplasmic transformation.

Wigglesworth later (1938) discovered that mosquito larvae hatched and kept completely under water for some days do not fill during the same stadium, even when returned to contact with air. From this and other considerations he concluded that the essential factor in normal filling was "secretion" of gas by the cells bounding the tracheae (these presumably being inactivated by long hypoxia in the submersion experiments). Gas secretion by hypothetical specialized cells or protoplasm was also invoked by Sadones (1895), Winterstein (1912), von Frankenburg (1915) in connection with this hydrostatic pressure theory, Pause (1918), and Akehurst (1922). A major objection to this proposal is the lack of any direct cytological, physiological and physical evidence of how secretion of gas might occur.

Keilin (1924) suggested that sudden absorption of tracheal liquid by the surrounding tissues (due to "imbibition or to a chemical reaction") would allow gas to diffuse from the tissues into the vacated space, provided the trachea could withstand a pressure of about an atmosphere. Bult (1939) similarly proposed imbibition as the explanation of the movements of tracheolar liquid and gas which Wigglesworth had attributed to osmotic forces. However, in neither Keilin's account nor in Bult's elaborate hypothesis is there any concrete evidence of the existence or nature of imbibitional changes.

An important characteristic of tracheal filling which must be accounted for by any proposed explanation is its great speed. It seems evident that at least the growth hypothesis of Weismann does not meet this requirement, and it has not been demonstrated that any of the other postulated mechanisms do.

The above review indicates that tracheal filling is far from being satisfactorily explained, and that it has not always been recognized that a process which might account for the appearance of gas in the tracheae would not necessarily explain the disappearance of liquid, or vice versa. The present investigation was undertaken in an attempt to clarify some of the problems outlined above.

Material and Methods

Living larvae of the mycetophilid fly *Sciara coprophila* Lintner were used usually at the beginning of the fourth stadium. The anatomy and development of the tracheal system, the process of molting and methods for raising larvae of known age have been described previously (Keister, 1948). Exposure to gases was carried out in brass cylinders each 22 mm. in diameter, 8 mm. high and with a wall 1 mm. thick. Each chamber had lateral inlet and outlet tubes and was cemented by one rim to an ordinary microscope slide. High humidity was maintained by lining the chamber wall with wet filter paper, and by bubbling the incoming gas through water. The larva was placed ventral side up in a minute droplet of water on a coverglass which was then inverted on the greased top rim of the chamber. Larvae so mounted could be observed for many hours, and at all magnifications. The work was done at $25 \pm 3°$ C.

For convenience in finding larvae in the proper stage, cultures were made up in petri dishes. Individuals about to molt are recognized by the fact that they stop locomoting and feeding and lie extended, usually with the anterior end raised, and make only slight and slow movements. Also, the tracheae are less distinct than in younger third instars due to the future fourth instar system enclosing them, and to the fact that usually much of the gas in the third instar system is replaced by liquid shortly before molting occurs. Further characteristic behavior usually involves: a period of axial back-and-forth rotation of the proventricular region of the gut; a period of slow, strong peristaltic waves of the body wall, alternately forward and backward; and finally, repeated bulging of the body just behind the head capsule. Soon thereafter the dorsal wall of the capsule and the cuticle just behind it split longitudinally, and the larva frees its anterior end and crawls forward out of the tubular exuviae, which are anchored to the substratum by viscid strands. Since the new tracheal system normally remains liquid-filled only for a short time after molting, larvae were mounted as quickly as possible after the splitting of the head capsule (usually within 1 minute). It was generally desirable to hasten the larva out of the cast by gentle prodding at the posterior end.

Observations

Normal tracheal filling

As described previously (Keister, 1948), visible gas is not present in the tracheae of newly-hatched (first stadium) Sciara larvae, and is found in only about one larva out of three even at the end of the stadium. When present, however, its extent is very constant. The actual filling process was never observed in spite of very numerous attempts. However, for reasons given in the earlier paper, it is considered unlikely that the gas enters by way of the posterior spiracles, which are the only pair present at this stage of development.

Second and third stadium larvae are less favorable for detailed study than fourth instars because of their smaller size. However, enough younger larvae were studied to show that tracheal filling after the first and second molts is essentially the same as described below.

Following the third molt, gas appears in 3 to 8 minutes and spreads throughout the (fourth instar) system in 1 to 2 minutes. The gas completely fills the principal

trunks almost instantly and apparently at a rather uniform rate. The lateral branches and spiracular connectives do not begin to fill until after the filling of the main trunks is complete, and they fill more slowly. Gas extends to the ends of the finest branches ("tracheoles"). Filling is initiated and progresses without any visible change in the normal locomotion and activity of the larva.

In normal larvae with spiracles in contact with air, careful and repeated observations under high magnification showed that gas first appears at some single point within the principal trunks, usually in one of the first four body segments. Although the suddenness of filling usually made it impossible to be absolutely certain of the exact spot where filling began, in a number of conclusive instances it began at some point other than a spiracle, and in no instance was it seen to begin at a spiracle. Ordinarily the gas spreads progressively and continuously both forward and backward from the starting point, and fills one main longitudinal trunk completely before crossing over by either the anterior or posterior commissures or both to spread through the tracheae of the opposite side. However, under conditions where filling has been interrupted experimentally (see below), it occasionally restarts in a new liquid-filled region, rather than continuing from its original stopping point.

Since the entire new system is still liquid-filled just after molting is completed, the tracheal liquid does not escape with the molted tracheae. Similarly, since the gas can be seen progressing distally into the liquid-filled branches (which end blindly), the liquid does not leave through any of the spiracles. This is particularly convincing after the first or second molt when the new system has only one pair of developed spiracles (anterior), and the gas can be seen passing posteriorly into regions where there are no possible exits. Furthermore, in larvae filling under oil (see below) no escaping aqueous liquid was seen. Also, liquid was sometimes seen to disappear from sctions of tracheae (usually the closed loops of the large lateral trunks) when gas approached simultaneously from both directions as Wigglesworth has also observed. From the above considerations it follows that the liquid passes through the tracheal and tracheolar walls into the blood and tissues as the gas appears.

Tracheal filling in submerged larvae

Since the open tracheal system of Sciara can, and probably normally does, fill independently of the spiracles, it was of interest to ascertain whether or not submerged larvae can fill their tracheae with gas. Of 27 late third instars submerged in a half inch of aerated water, 21 succeeded in molting in the course of 2 days, although they were not able to free themselves completely from the old cuticles for lack of a relatively dry surface for attachment. The tracheae of these larvae became gas-filled except for occasional individual tracheoles or segmental units. Normally no gas passes from the old to the new tracheae; but even if it did under the abnormal circumstances of the test, the amount would be far too small to fill the fourth instar system. As a possible indicator of whether the gas comes from an internal or external source, the experiment was repeated with freshly-molted larvae (i.e., with completely liquid-filled tracheae) taking care to exclude all air-bubbles from the dishes. Twenty or more larvae were used in each of three liquids: ordinary water,

freshly boiled water (the containers being filled to the top and covered), and a 6 mm. layer of mineral oil. In every instance filling began in approximately normal time and proceeded in the usual fashion. These experiments indicate that a visible external gas bubble is not necessary to initiate tracheal filling, in agreement with the previously described observations under high magnification. Furthermore, since the amounts of dissolved gas were probably quite different in the three liquids, the results suggest first that the gas which fills the tracheae of submerged larvae comes from some internal source, and second that filling is independent of O_2 tension over a wide range.

Tracheal filling in gases other than air

To investigate further the nature of the tracheal gas and the role of O_2 in filling, fresh ecdysiasts were exposed to commercial N_2, CO_2 and CO [1] in the gas chambers previously described.

Although there were considerable variations in the individual responses, the larvae were completely immobilized by a few minutes' exposure to any of the gases. In most instances gas appeared in portions of the main tracheae either in normal time or within 30 minutes and usually after the larvae were motionless. However, filling was not completed unless air was admitted to the chamber. Filling could be stopped and restarted repeatedly by alternating exposures to the tank gas and to air.

If the gases used were passed over hot copper gauze before admission to the gas chamber, filling was completely inhibited for an indefinite period (2 hours was the longest exposure tried). If the larva was returned directly to air after a period of complete anoxia not exceeding 75 minutes, filling usually was normal. Longer periods of complete anoxia often led to incomplete or delayed filling, and sometimes to complete and permanent inhibition. Permanently affected larvae died after a few hours, though they might, for a time, resume body movements, gut peristalsis and heartbeat. Filling was not visibly affected by pure medicinal O_2.

The quantitative relations between tracheal filling and O_2 tension, and differences between the specific effects of the individual gases, will be reported in detail in another communication, but for present purposes it may be said that, if given initially, mixtures of 0.3 per cent O_2 and 99.7 per cent CO_2, CO or N_2 suffice to permit gas to appear in the liquid-filled tracheae.

Effect of low temperature on tracheal filling

In freshly-molted larvae placed in small droplets of water on glass kept on melting ice, filling was indefinitely delayed (2¼ hours was the longest exposure tried). When returned to 25°, filling was normal but could be halted and restarted repeatedly by alternately chilling and rewarming the larva. During the exposure to 0° the larvae were practically motionless but responded markedly to mechanical stimulation.

[1] No difference was observed between experiments done in the light and those done in the dark, using a deep red filter to examine the larvae.

Experiments with hydrostatic and osmotic pressure

Attempts were made to prevent filling by exerting pressure from a coverglass on larvae mounted on a slide either in water or in air. It was found impossible to prevent filling from starting even with the greatest pressure which could be applied without bursting the larva.

The larval cuticle of Sciara is somewhat permeable to water, and exposure to hypertonic solutions causes withdrawal of water from the body. Since the consequent lowering of body turgor might accelerate filling, if release of hydrostatic pressure were a factor in filling, freshly molted larvae were immersed in double Ringer's solution. Filling was normal.

Discussion

From the foregoing experiments the following deductions can be made concerning some of the previous explanations of tracheal filling: (1) Tillyard's hypothesis necessitates a gradient between body and tracheal liquids of CO_2, a gas with high aqueous solubility and diffusion coefficient. This would be rather improbable even for larvae in air, but seems quite out of the question in Sciara larvae, in view of their demonstrated ability to fill in 99.7 per cent CO_2. (2) The fact that filling does not occur in larvae completely relaxed by anoxia argues against mechanisms involving a fall in hydrostatic pressure, as do the experiments with coverglass pressure and hypertonic solution. Conversely, the fact that filling can occur in larvae motionless in 99.7 per cent CO_2 runs counter to Wigglesworth's claim that CO_2-narcosis *per se* inhibits filling, and to Standtman-Averfeld's activity theory. Muscular activity as a factor also seems to be ruled out by the lack of filling after the body contractions induced in chilled Sciara larvae by prodding. (3) The inhibition of filling by anoxia argues against both osmotic pressure and imbibition as prime factors in filling, since both are claimed to be enhanced by anoxic catabolism. (4) Weismann and Keilin believed that the tracheoles were the site of absorption of tracheal liquid, but our findings that liquid can leave through trunk walls and that the trunks fill completely before gas enters any of the side branches indicate that the filling mechanism also operates through the main tracheae. This is supported also by the apparently rather uniform rate of filling in the main trunks (where a deceleration would be expected if the liquid were leaving via the side branches). An additional puzzle is that most reports agree that the largest trunks, which have the highest ratio of liquid content to surface area, fill in a matter of seconds; whereas, the fine, thin-walled branches, where it seems that imbibition, osmosis, etc. should be more effective, fill much more slowly. (5) The fact that the tracheal filling of the first instar does not occur for a day or more after hatching (if at all), whereas tissue osmotic pressure might be expected to be highest during the struggles of the embryo at hatching, militates against the osmotic theory. (6) From the fact that the Tenebrio embryo can fill its tracheae either with outside air or with gas derived from tissue fluids, Sikes and Wigglesworth concluded that there is no essential difference between filling in closed and in open systems. Although this conclusion does not appear justified logically, our finding that Sciara. which has an open tracheal system, normally may fill its tracheae with gas from some internal source, suggests that other insects (e.g., Tenebrio) likewise normally may fill as if they had closed systems in spite of opportunity to take in outside gas. (7) Our

demonstrations that (at ordinary temperatures) O_2 is an absolute prerequisite for tracheal filling, and that filling may be delayed by O_2-poor conditions, offer a possible explanation for the observations of Sikes and Wigglesworth that the Lucilia embryo will fill under water, but only if near the surface; of von Frankenberg that Corethra will not fill its tracheae if kept in boiled water; and of Tillyard that dragonfly larvae in O_2-poor water filled slowly. (The first observation was not elaborated by the authors; the second was attributed to insufficient dissolved gas in the surrounding water to serve for filling the vesicles; and the third to "weakening" of the larvae.) (8) The strict O_2-dependence of the initial filling of tracheae makes it questionable whether the "metabolic" movements of tracheolar liquid and gas studied by Bult are brought about by the same mechanism (though both processes are CO-insensitive) since Bult's postulated imbibitional mechanism is apparently anaerobic.

The net result of the above discussion is to eliminate from serious consideration as prime factors in tracheal filling all proposed mechanisms except those postulating "secretion" of gas. As previously stated, however, this concept is so vague that no critical consideration of it is possible at present. Insofar as "secretion" is a metabolic phenomenon, it is compatible with our finding that tracheal filling is absolutely dependent on O_2 and can be indefinitely inhibited by low temperature. However, we have no evidence as to what metabolic change might be involved, except that it is unlikely to be mediated by the cytochrome system. Numerous other aerobic processes (not all necessarily metabolic) are conceivable. Tillyard for example regarded O_2 as essential for a metabolic process by which gases dissolved in the external medium were transferred into the body and liberated in the tracheae.

It might, of course, be assumed that the *initiation* of filling and the actual filling process depend on quite different mechanisms. One could imagine for example that the reaction which requires O_2, and which might be metabolic, simply pulls a trigger which sets off a physical process resulting in tracheal filling. A number of possible mechanisms will be dealt with in another communication, but the evidence already available permits several deductions. Keister (1948) reported that scraps of air-filled third instar tracheae sometimes break off and are left within the new system at molting. The fact that filling will not start, or if previously started will not continue, in such larvae in the absence of oxygen or near 0° C. shows that filling does not progress automatically once gas bubbles are present in the tracheal liquid. The further fact that filling, when it occurs, does not necessarily begin in regions containing tracheal scraps, shows that preformed gas bubbles are not a prerequisite for the initiation of filling. The same conclusions follow from the fact that after filling has been interrupted experimentally it sometimes resumes in a new (liquid-filled) region rather than continuing from its original stopping point. Finally, since filling will occur in gases ranging from tank O_2 to practically pure CO_2, CO or N_2 it is very unlikely that either the initiation or the progress of filling depends on the attainment of any critical ratio between gas concentrations, or upon the presence of any specific gas (except O_2).

Summary

(1) After each molt the new tracheal system of a Sciara larva in air normally remains filled with liquid for 3 to 8 minutes. It then fills spontaneously, rapidly

and completely with gas, beginning at some point in a main trunk. The gas comes from some internal source.

(2) Sciara larvae can molt under aerated water, and can fill their tracheal systems with gas while completely submerged in aerated or boiled water, or mineral oil.

(3) Tracheal filling can occur if the larva is in a mixture of 99.7 per cent CO_2, CO or N_2 with 0.3 per cent O_2, but not in complete absence of O_2.

(4) Tracheal filling is indefinitely inhibited near 0° C.

(5) Filling may be stopped and restarted repeatedly by alternating exposures to anoxic gas and air, or to low and room temperatures.

(6) Initiation and progress of tracheal filling are apparently independent of body movements, body hydrostatic pressure, and critical gas ratios.

(7) It is suggested tentatively that tracheal filling involves a metabolic process.

(8) A review and critique of previously proposed mechanisms of tracheal filling is presented.

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AN ABBREVIATED CONJUGATION PROCESS IN PARAMECIUM TRICHIUM

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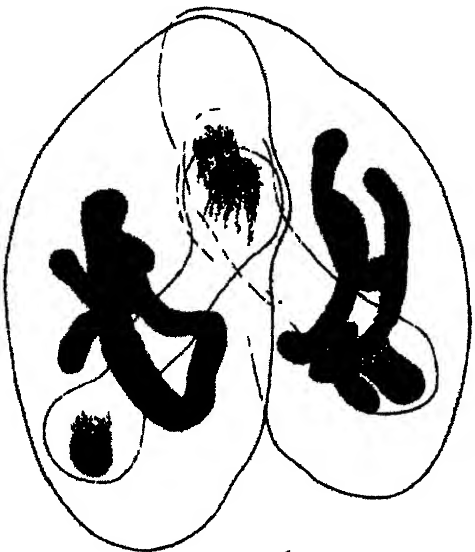
The remarkably constant, well-ordered, complex series of nuclear processes which are characteristic of ciliated protozoa during conjugation has been established by a host of cytological investigators. The almost monotonous regularity of the maneuvers in many species of ciliates during conjugation has led to a fairly stereotyped concept of the events of conjugation in ciliates generally; three pregamic divisions producing the pronuclei (with degeneration of nuclei after the first and/or second divisions), interchange of gametic nuclei, fertilization, and the reorganization of a new nuclear complex from the synkaryon after a characteristic number of divisions. The invariability of this "standard" process was called into question recently by a number of investigators including the author (Diller, 1936), who suggested that conjugation might not always involve an exchange of pronuclei and reciprocal fertilization, but fusion of pronuclei arising in the same member of the pair (autogamy). Both cytological (Wichterman, 1940; Chen, 1946; and Diller, 1948) and genetic (Sonneborn, 1947) studies have subsequently demonstrated the reality of autogamy in conjugation. Moreover, genetic effects due to cytoplasmic interchange during conjugation have been claimed by Sonneborn (1943, 1945) and Dippell (1948). Another event in the classical picture of conjugation—the puzzling third pregamic division—has now been shown to be not indispensable. In certain races of *P. trichium* (Diller, 1948) the conjugants may omit the third division and proceed with either reciprocal fertilization, autogamy ("cytogamy" of Wichterman) or parthenogenetic development of gametic nuclei.

In view of the great versatility of nuclear behavior shown by *P. trichium* during conjugation (Diller, 1948) and the large favorable micronuclei which this species possesses, it would seem to be of interest to describe a further variation from the "standard" conjugation behavior. In this heretofore undescribed process certain stages are eliminated and the micronuclei proceed directly and without degeneration of their products to establish a new nuclear apparatus.

Source of Material and Techniques

All the material on which the present study was made, was derived from a pond collection kindly furnished by Dr. Hannah Croasdale of the Department of Zoology, Dartmouth College. The collection was taken on September 20, 1946, from a pond on Dr. Carleton's grounds in Hanover, N. H. Some of this material was introduced into hay infusion on September 23, 1946, and on the next day about fifteen pure-line mass cultures, each descended from a single animal, were isolated from this culture. Several small mass cultures were also established at this time. No

PLATE I



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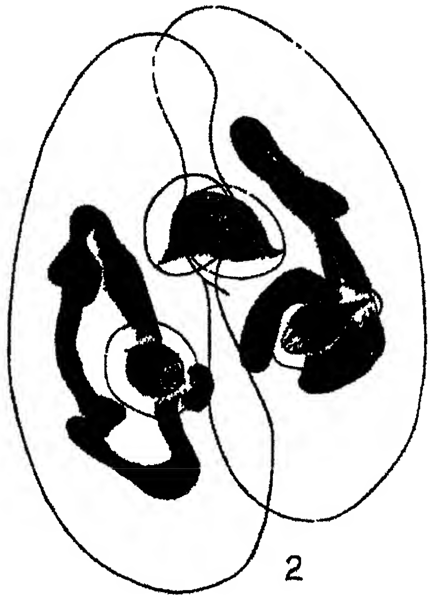

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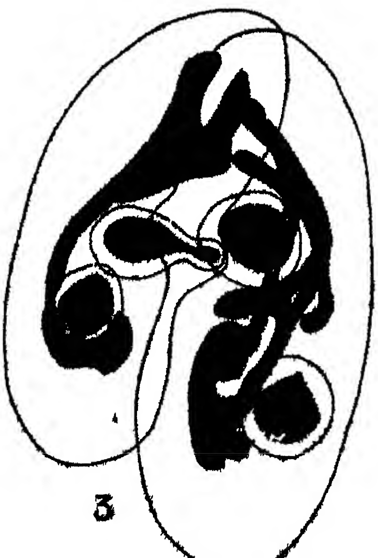

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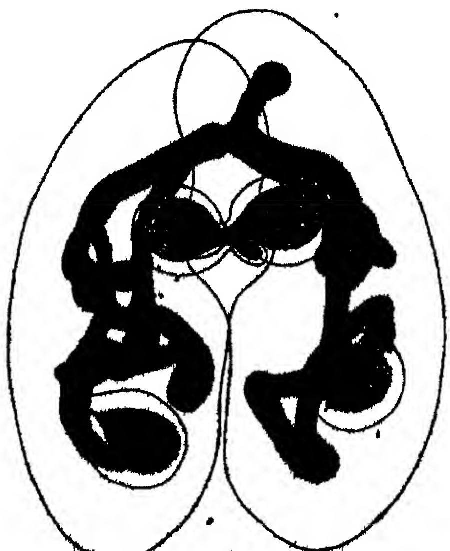

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significant differences in cytological behavior between the various lines were noticed, although most of them were examined from time to time and the selection of material to be studied was more or less random. Very shortly after their establishment, conjugation occurred in many of the cultures; for instance, in isolation culture No. 10, conjugation was in progress by October 3.

In most of the lines conjugation occurred in at least small numbers, at all times. The cultures were maintained on hay and malted milk, boiled in pond water. Several of them (isolation cultures Nos. 5 and 6) were mixed. Although there were small numbers of conjugants in each culture at the time of admixture, the combination resulted in a rather heavy incidence of conjugation so that it would seem as if these two cultures may have been opposite mating types. The cultures were maintained until June, 1947, when they were abandoned. Toward the end of their life span the cultures showed a more conventional behavior and finally did not conjugate at all. Temperatures in the laboratory became rather high and this may have been responsible for the decline of the cultures.

All the observations reported in this paper were made on killed and stained material. The animals were pipetted from the cultures into a centrifuge tube, concentrated, allowed to stand for a few minutes, and then fixed in Perenyi's fluid. They were sometimes subsequently treated with Schaudinn's fluid, and stained in acetic orcein or in Grenacher's alcoholic borax carmine. Both stains gave very good results. Usually fast green or indulin were used as counterstains. All the technique was carried out in the centrifuge tube and the animals mounted on slides in diaphane or clarite.

EXPLANATION OF PLATES

These are camera lucida drawings of stained whole animals from six isolation cultures started Sept. 24, 1946, and a small mass culture started Sept. 28, 1946, all derived from Carleton Pond, Hanover, New Hampshire Magnification about 1200 times. All the figures illustrate *Paramecium trichium* during "abbreviated" conjugation The animals were fixed in Perenyi's fluid and stained with Grenacher's alcoholic borax carmine or acetic orcein and counterstained with indulin or fast green The specimens shown in Figures 1, 2 and 9 are representatives of small mass culture A; Figure 3, isolation No 15; Figures 4 and 5, isolation No. 10; Figures 6, 7, 10, 11 and 16, isolation No. 14; Figure 8, isolation No. 6; Figures 12 and 15, isolation No. 9; Figures 13 and 14, isolation No. 17. Only the nuclear structures have been drawn. In some of the figures, particularly the later stages, old macronuclear fragments lying on top of the structures which were intended to be illustrated were omitted for the sake of clarity.

Plate I

Explanation of Figures

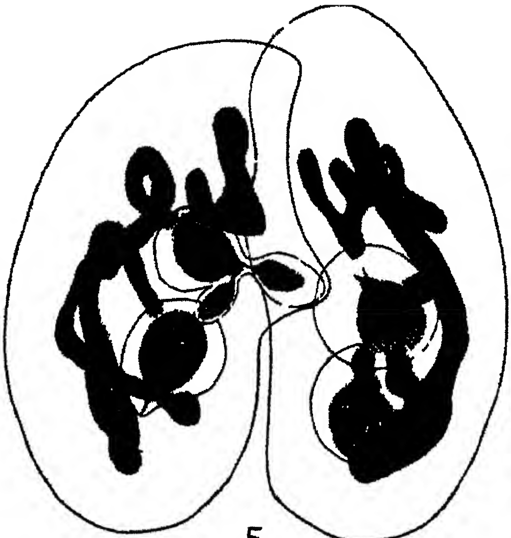
Figure 1. Telophase of first maturation division in a culture engaging in "abbreviated" conjugation. The macronuclear skeins (simple) seem to develop earlier than they do during "standard" conjugation. A daughter nucleus is found in the paroral cone region of each conjugant

Figure 2. Two nuclei resulting from the first division. Each has a tail indicative of recent separation.

Figure 3. Two nuclei in each conjugant. Twisted chromosomes and a knob on the nucleus in the paroral cone region of the left conjugant are suggestive of its impending passage. Since the corresponding nucleus in the right conjugant is not in a similar condition, this pair suggests a one-way passage.

Figure. 4. "Migratory" nuclei produced after the first division. A nipple-like process on each extending toward external boundary of cone. Suggestive of reciprocal transfer.

Plate II



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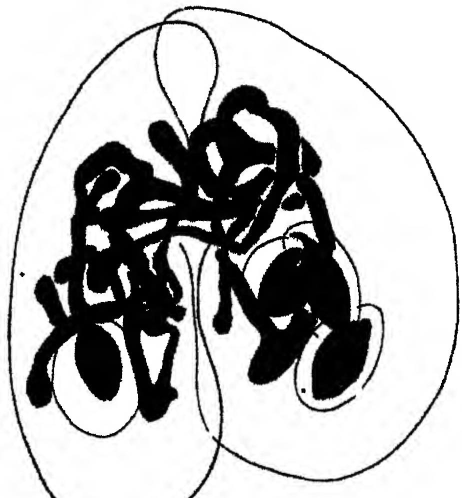

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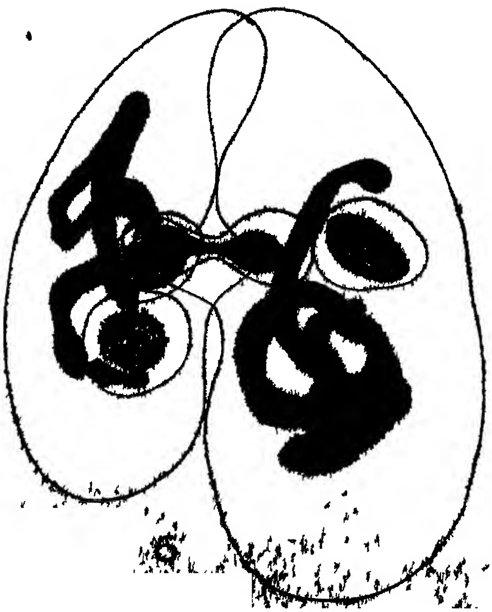

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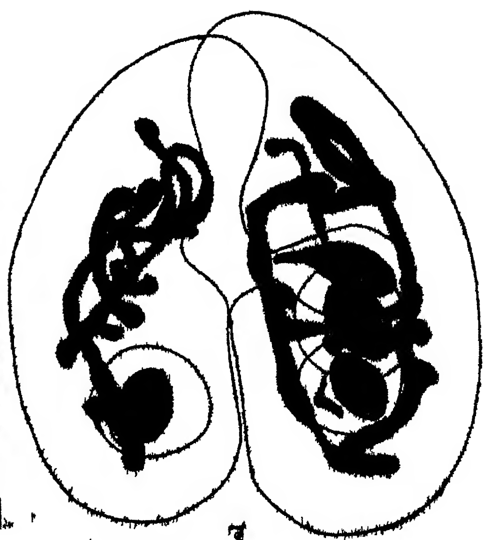

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Observations

Although no observations were made on the length of time that the members of the pair remain attached in abbreviated conjugation, it is my distinct impression that the time is much less than for the conventional method. It is probable that the first division of the micronucleus consumes less time than ordinarily is the case. The macronucleus in abbreviated conjugation seems to be somewhat precocious in its skein formation By the time of the telophase of the first division, a simple macronuclear skein (Fig. 1) has formed. One sister chromosome group of each spindle is likely to be found in, or near, the paroral cone. The daughter nuclei, immediately after separation, frequently have "tails" on them (Fig. 2). This is true also for the corresponding post-telophase stage of the other divisions. Normally, the first division is not followed by degeneration. I have seen only one or two pairs, in abbreviated conjugation, in which degeneration of nuclei was evident. This is rather unusual because in conventional conjugation, as well as in the type in which only the third pregamic division is omitted, degenerating nuclei after the first and second divisions are the rule.

A reorganization of the two nuclei in each conjugant leads to a premetaphase condition (Fig 3 and others). A knob-like, nipple-like, or handle-like process (Figs. 3, 4, 5, 6, 7, 8 and 10) is formed on one (Fig 3) or two (Fig. 4) of the nuclei. This modification marks the nucleus as a potential migratory gametic nucleus Undoubtedly, it is reflective of cytoplasmic stresses, pressures and/or currents in the cone regions. The narrow pointed process may be directed toward the cell boundary or toward the interior of the cell. The chromosome threads are frequently arranged in a spiral fashion, suggesting a twisting influence on the nucleus. In Figure 3, the presence of a single nucleus in the paroral cone of the left conjugant, with a terminal knob, and the absence of a similar structure in the right conjugant suggest an imminent one-way passage. Frequently, the macronucleus of one conjugant is a little more advanced in skein formation than is the other (Fig. 3). In contrast, two such migratory nuclei (Fig. 4) may be present, indicating an approaching reciprocal transfer.

Occasionally the pinching effect appears to be so severe as to cause a disruption of the migratory nucleus into several parts. Such an instance is represented in Figure 5. It is conceivable that this process may be the means whereby small accessory nuclei arise, by a purely amitotic mechanism. This possibility will be considered later in connection with subsequent stages. It is probable that most of these constricted nuclei would recondense and adjust to the normal condition after

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Plate II

Explanation of Figures

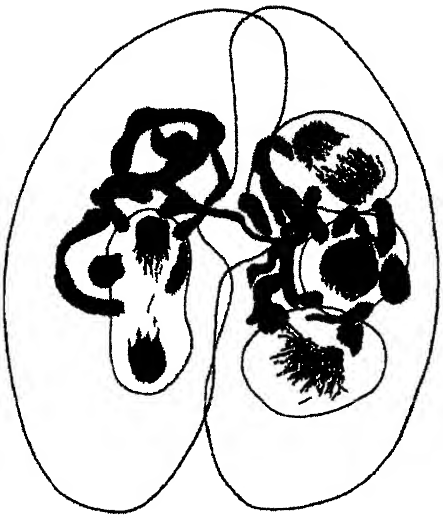
Figure 5 The "migratory" nucleus of the left conjugant pinching off two small portions, each connected to the larger section. One of the small accessory nuclei is lying in the cone.

Figure 6. Macronuclei in simple skeins. No degenerating micronuclei. Migratory nucleus, constricted in the middle, passing through the cone from the right conjugant to the left. One-way passage.

Figure 7 One-way passage of migratory nucleus, after first division, from the left conjugant to the right one. Tail of migratory nucleus still in tip of cone.

Figure 8. One-way passage of a migratory nucleus. Interchange of rather coarse macronuclear strands in both directions.

PLATE III



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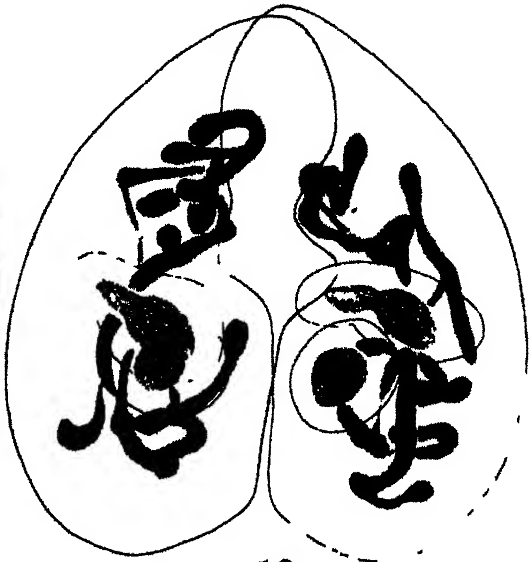

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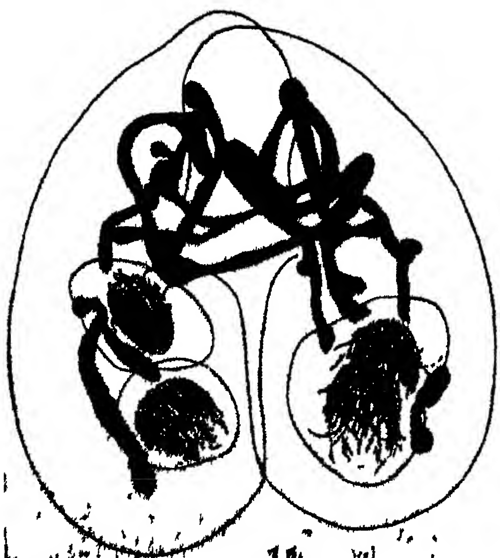

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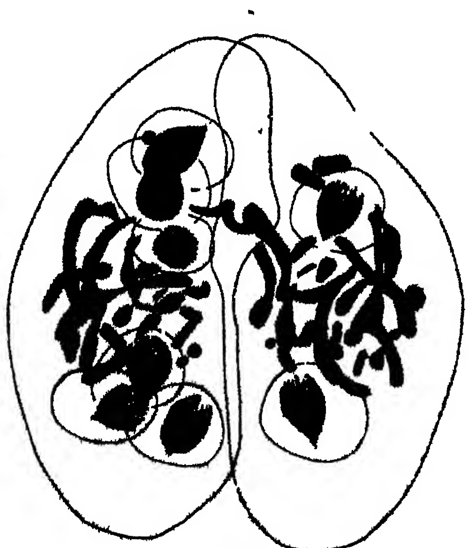

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the temporary stresses had been relieved. I have seen one other instance, not figured in this paper, which could produce a similar result. One member of the pair had two normal nuclei from the first division. The other had a tripolar telophase of the first division. One of the three sister chromosome groups was smaller than the other two.

At the time when the migratory nuclei are actually passing through the paroral cones they often show an equatorial attenuation. Such a dumbbell effect is shown in Figure 6. The migratory nucleus is passing into the small left conjugant. There is no indication of nuclear passage in the reverse direction. The macronuclear skeins are still relatively simple and coarse. In this case there is cytoplasmic continuity at one level only. Figure 7 illustrates a slightly later stage of one-way passage of a migratory nucleus after the first division. Its tail is still in the cone region. Probably in this pair there is cytoplasmic continuity between the conjugants at two levels.

It is difficult to ascertain the frequency of the occurrence of the different modes of behavior of the nuclei, after the first division, in the Carleton Pond stock of *P. trichium*. One-way passage of a nucleus, leading to the spectacular unbalanced condition of three nuclei in one member and one nucleus in the other conjugant—a situation which first attracted the author's attention to this process—is by no means a rarity in these stocks. Two-way passage (interchange), as suggested by Figures 4, 10 and others, is also quite common. In the event of an original heteroploidy of the micronuclei of the two conjugants, it is possible to determine at a later stage whether interchange had occurred. A third alternative is evident: the failure of nuclei in both conjugants to migrate and their development in the same conjugant in which they arose. This possibility is, in the author's opinion, a valid one, but seems to be more rare than the other two. Apparently, breakdown of the tips of the paroral cones, cytoplasmic currents, and/or internal pressures at the proper stage are the factors which determine the movements of nuclei at this time. The nature of these forces is entirely conjectural but it is of interest to note that they may be unequal in the two members of the pair.

Shortly after the passage of the micronuclei, or their non-passage, strands of the macronuclear skein may, or may not, become stretched across the cone regions from one conjugant to the other in much the same fashion as in unabbreviated conjugation (Diller, 1948). Passage of the macronuclear skein may be unidirectional

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PLATE III

EXPLANATION OF FIGURES

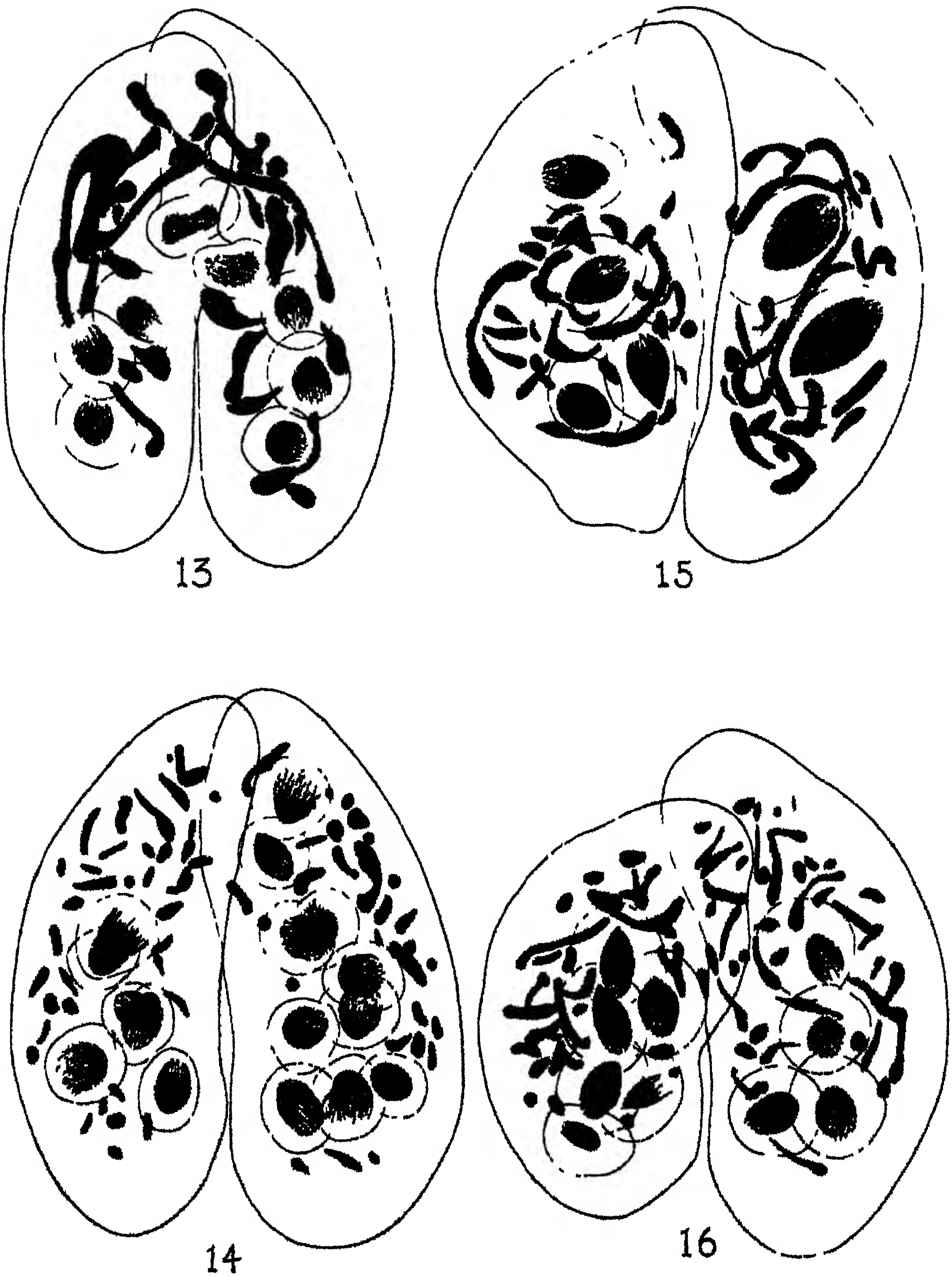
FIGURE 9. One micronuclear figure in left conjugant; three dividing nuclei in right conjugant Second division. Macronuclear strand passing from left conjugant into right. All the nuclei are in slightly different mitotic stages.

FIGURE 10. Two gametic nuclei in each conjugant. Either cross-fertilization, autogamous fertilization, or parthenogenesis, is imminent. Uncertain whether there is cytoplasmic continuity in oral cone regions. The "migratory" nuclei are retaining the constrictions characteristic of the migratory condition.

FIGURE 11. Synkaryon formation in right conjugant. Two separate nuclei in left conjugant. In the latter, either nuclear fusion is delayed or the gametic nuclei are going to develop parthenogenetically. Macronuclear interchange in both directions.

FIGURE 12. Six nuclei in left conjugant; two in right conjugant. Macronuclei in short complicated strands.

PLATE IV



(Fig. 9) or may extend in both directions (Figs. 8 and 11). Macronuclear exchange was very frequent in the Carleton stock. In case it is unidirectional, the strands can pass either from the uninucleate conjugant into the trinucleate member, as in Figure 9, along the path which the single migratory nucleus took, or in the reverse direction. The direction of macronuclear movement seems not to be directly correlated with the direction of micronuclear movement. Exchange of macronuclear material apparently marks the end of interconjugant micronuclear movement.

As remarked above, there is normally no degeneration of nuclei at this stage, or any other stage, in abbreviated conjugation. One can detect several alternative modes of behavior of the nuclei from this point, keeping in mind the possibilities that the nuclei of one member of the pair may be behaving differently from those of the other member, and even that the nuclei in the same conjugant may be diverse in their activities. First, fertilization (synkaryon formation) may occur. Such a condition is shown in the right conjugant of Figure 11 and such was probably the ancestry of the two nuclei in the right member of Figure 15. Depending on whether interchange had occurred, cross-fertilization or self-fertilization would be accomplished. Second, parthenogenetic development of the nuclei may take place. This seems to be the most frequent type of activity in abbreviated conjugation. Third, combinations of fertilization and parthogenetic development may be adopted. Although the critical stages are rare, it is possible by reason of size differences to reconstruct previous history.

Figure 9 illustrates the micronuclear activity of the second division. The conjugant on the left contains a late anaphase micronucleus, while that on the right has three dividing nuclei in slightly different mitotic stages. It is a little unusual for the micronuclei to show such asynchrony. Probably the two anaphase nuclei, one in each conjugant, are sisters. It seems likely that all of these nuclei are developing without fertilization (parthenogenetically).

It is difficult to be sure about the exact history and the immediate fate of the nuclei of Figure 10. The "tailed" nuclei may have been interchanged, or not, and may be on the point of fusion with the stationary nuclei. Otherwise, parthenogenetic development would be expected to follow.

Figure 11 shows synkaryon formation in the right conjugant and two separate

Plate IV

Explanation of Figures

Figure 13. Unusual interchange at the end of the second division, with no degenerating nuclei apparent. The nucleus at the top is pressing against the cone moving toward the left, while the nucleus directly below it is part-way through the cone, passing into the right conjugant.

Figure 14. Six nuclei in left conjugant. Ten nuclei in right conjugant. Presumably, this condition arose from a one-way passage of a gametic nucleus at the stage represented in Figure 13. Old macronuclei represented by closely packed short rods and spheres (many omitted).

Figure 15. Four nuclei in left conjugant. Two very large nuclei in right conjugant. Probably parthenogenesis has occurred in the left conjugant while synkaryon formation has taken place in the right member.

Figure 16. Four nuclei in right conjugant. Four large nuclei and two small ones in the left conjugant. The latter may have arisen from pinched-off parts of nuclei after the first division, as suggested by Figures 3 and 5. (They may have originated in the right conjugant).

nuclei in the left conjugant. In the latter, either nuclear fusion is delayed, or the gametic nuclei are going to develop parthenogenetically. The latter alternative seems to me the more probable, since my observations suggest that very little time elapses before nuclear fusion is completed.

Occasionally I have found that one or both of the conjugants at later stages possess nuclei of different sizes. Aside from the explanation of original heteroploidy of the conjugants, this can best be interpreted by assuming that the larger nuclei have arisen from synkarya, while the smaller ones have developed parthenogenetically. Assuming that the number of nuclear generations is the same in both conjugants, synkaryon formation in one and not the other will result in different numbers of nuclear products in the two members at later stages (cf. Fig. 15). Although this is not the only explanation, I believe that asymmetric synkaryon formation is a valid one. One wonders whether an extra postzygotic division is required for final reorganization since there has been a reduction in nuclear number in the conjugant which produced a synkaryon. Another device for bringing about unequal nuclear numbers in the two conjugants is for the mitotic stages to become slightly out of step with each other. I believe this to be a real, but rather rare, happening. However, in the uninucleate-trinucleate pairs the single nucleus seems often to be ahead of the three others (Fig. 9).

Figure 12 shows completion of the second division in a pair in which there has been one-way transfer. By this time the macronucleus has usually fragmented into complicated short strands and rodlets and no longer can be traced to the opposite cell. Apparently the paroral cone intercommunications heal over at this time. The conjugants separate after this stage or during the next (third) division.

Migration of nuclei in abbreviated conjugation is not completely restricted to the time immediately after the first division. Very rarely, interchange can occur after the second division. Two such cases are shown in Figures 13 and 14. In the former, each conjugant has four nuclei, one of which is located in the paroral cone and is projecting into the other animal, apparently on the verge of effecting interchange. A one-way transfer of this type would result in five nuclei in one conjugant and three in the other. At the conclusion of the third division of such a hypothetical case, six and ten nuclei, respectively, would be found in the conjugants. That is apparently the explanation of the asymmetric condition of the pair illustrated in Figure 14.

A rather unusual and interesting asymmetrical case is illustrated in Figure 16. Four nuclei, following the second division, are present in each conjugant. In addition, there are two small nuclei in the left conjugant. These may have arisen as "buds" pinched off the nucleus at the time of the first division, as suggested earlier, which have persisted through division. (See Fig. 5.) They seem perfectly viable and normal.

The events subsequent to the third division, when the animals separate, have offered no special points of interest. Presumably reorganization, anlagen formation, and disappearance of the old macronucleus are similar to the standard processes characteristic of *P. trichium* (Diller, 1948), although I have made no particular effort in these studies to work out the post-conjugant stages. Regularly, the mature exconjugants would be expected to have four macronuclear and one micronuclear anlagen.

Discussion

The abbreviated conjugation process in the Carleton race of *P. trichium*, reported in this paper, accomplishes the ends of nuclear reorganization in a remarkably simple and direct manner without wastage of micronuclei and without unnecessary stages. However, it is so unorthodox and so divergent from the conjugation pattern of other ciliates, and even of other races of the same species, as to pose problems about its general significance, and, in fact, about the meaning of certain phases of the conjugation process as a whole. The standard or conventional scheme of micronuclear activity in the ciliates involves three pregamic divisions (two have been reported in a certain race of *P. trichium*, Diller, 1948), and a variable number of postzygotic divisions which reconstitute the definitive nuclear complex. The term "postzygotic divisions" is here extended to include parthenogenetic divisions or generations as well as those of fertilization nuclei. It is borne in mind, of course, that variation in numbers of macronuclear and micronuclear anlagen is common but is fairly constant for a given species. In *P. trichium*, in the standard process, there are two or three pregamic divisions and three postzygotic divisions. In abbreviated conjugation three divisions, simply, are required to complete the process. (Possibly an extra division is appended in case synkaryon formation is involved.) Similar numbers of final nuclear products arise in both processes. The failure of nuclei to degenerate in the abbreviated process, generally, accounts for the end products being the same in number as in standard conjugation. The question then arises as to the homology of the nuclear generations in abbreviated conjugation with those in the standard process. In both, the first division shows a characteristic polarized (not crescentic) prophase stage. This may well be indicative of a maturation or a reductional process and is followed, in the standard conjugation, by one or two other divisions before fertilization or parthenogenetic development. However, in abbreviated conjugation there is no further division before nuclear exchange and fertilization (or parthenogenesis) are accomplished. If one were to assign the exchange period as a central reference point in both processes, then one can consider the first division in abbreviated conjugation as a maturation division and the second and third divisions as being homologous with the postzygotic divisions of the standard process. If this interpretation is valid, what can be inferred about the chromosomal cycle in abbreviated conjugation? A comparable problem was raised before (Diller, 1948) in connection with the omission of the third division in certain races of *P. trichium* and the parthenogenetic development of reduced nuclei; it was concluded that under these circumstances each conjugation would be expected to result in a progressive diminution of chromosome number. Unfortunately, direct observation of chromosome numbers in the various generations is very difficult, if not impossible, to make, and even estimates of nuclear size are not very satisfactory in spite of the large and comparatively favorable micronuclei of *P. trichium*. It has been considered axiomatic that two maturation divisions are necessary to bring about chromosomal segregation and reduction in mature gametes. This is undeniably accomplished in the standard conjugation process, even when the third pregamic division is omitted, but is doubtful in abbreviated conjugation.

Two possibilities suggest themselves. First, that reduction is completed in the later divisions and that the final nuclei are haploid, unless fertilization occurs. In

the latter eventuality, the awkward situation of the occurrence of a maturation division before fertilization and another after fertilization would exist. A second, and more probable, speculation is that the gametic nuclei are not reduced but diploid, and the nuclei arising by parthenogenesis would remain diploid while those derived from synkaryon formation would be tetraploid (cf. Fig. 15). Although a good deal of heteroploidy was evident in these cultures, hypoploidy was not nearly as extreme nor as conspicuous as in certain other stocks which I have been studying.

In correlation with the shortened morphological manifestations, it would be interesting to know how the time relationships of abbreviated conjugation compare with those of the standard process. I have the impression that abbreviated conjugation takes a shorter time than the standard process, but no positive evidence on this point. Unfortunately, the cultures were discarded before this information was obtained, in fact, before it was realized that abbreviated conjugation was happening; and I have not been able to secure any more stocks from the Carleton Pond, although several collections were made. The causes of the induction of abbreviated vs. standard conjugation are also entirely unknown at present. It seems to be not entirely a racial or genetic effect, since there were some instances of standard conjugation in certain of the Carleton Pond stocks.

I know of no other conjugation study in ciliates in which nuclear transfer has been observed at the end of the first division. The mechanism of conjugation activity has apparently been accelerated to bring about nuclear passage two generations ahead of the time usually required: the tips of the paroral cones have broken down and the macronuclear skein is far advanced. The latter seems to be precocious and attuned to the prospective activity of the micronuclei. Transfer of the micronuclei may be unidirectional, resulting in the asymmetrical condition of one nucleus in one member and three in the recipient, or reciprocal (interchange), or, probably, there can be non-passage. Such a selection suggests a chance determination. A pinching or constriction of the "migratory" nucleus before and during passage may be extreme —so severe as to cause a complete separation of fragments from the nuclei. These may persist and continue an apparently independent existence. (One hesitates to apply the terms "migratory" and "stationary" nuclei to the products of the first division, with the implication that these are reduced nuclei and that they invariably are involved in interchange.)

As in other accounts of conjugation in *P. trichium* (Diller, 1948), macronuclear passage may occur in abbreviated conjugation, after micronuclear migration. The macronuclear exchange may be either reciprocal, unidirectional, or, probably, omitted. In case of unidirectional micronuclear passage, macronuclear exchange is not necessarily along the same path, i.e., from the conjugant with one nucleus into the one with three nuclei, but may be in the opposite direction from the trinucleate to the uninucleate conjugant.

Also, as in other processes of conjugation in *P. trichium*, the subsequent micronuclear activities may be variable: fertilization (cross-fertilization or autogamy), parthenogenetic development, or combinations of fertilization and parthenogenesis in the two members of a pair or even, probably, in the same member of the pair. The versatility, lability and variability of micronuclear activity in *P. trichium* should be susceptible to experimental attack and analysis.

Summary

1. A process of "abbreviated" conjugation occurs in one race of *P. trichium* in which the number of micronuclear divisions is reduced to three (or possibly four) from the "standard" pattern of five or six.

2. There may be exchange of micronuclei at the conclusion of the first division. Frequently, unidirectional passage of a gametic nucleus occurs at this time so that an asymmetry results in the two conjugants, one of them having three micronuclei and the other conjugant one micronucleus.

3. The products of the first division proceed, directly, to reconstitute the new nuclear apparatus. This they do by synkaryon formation, parthenogenetic development or a combination of the two, usually dividing twice. There is no degeneration of nuclei between divisions.

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ADDENDA

Seminar, July 12, 1949, Marine Biological Laboratories

X-ray mutations and fecundity of Mormoniella. Marion E. Kayhart and P. W. Whiting.

Females were treated (4000–8000 r) and mated to untreated males. Lowest dose given is, by analogy with Habrobracon, well above lethal for metaphase eggs. Therefore all offspring must be produced from prophase or preprophase. Fecundity tests show decrease in offspring with increasing dose given to adult females—(1173/10) 117.30 per untreated female, (229/27) 8.48 per 4000 r treated, (91/40) 2.27 per 5000 r, (199/88) 2.26 per 6000 r, (105/86) 1.22 per 7000 r, (69/83) 0.83 per 8000 r. Treatment (4000 r) of middle-aged pupae is more effective—(26/40) 0.65 offspring per female—than of young pupae—(113/50) 2.26—, old pupae—(445/80) 5.56—, or adults. Treatment (6000 r) of middle-aged pupae is more effective— (36/40) 0.90 per female—than of old—(88/40) 2.20—or adults. This sensitivity of eggs of middle-aged pupae is probably due to injury to nurse and follicle cells, as well as to oocytes.

Among 1262 sons of treated mothers were five independently occurring eye color "mutants" —scarlet-2 (found dead), scarlet-3 (overetherized), pinkish (sterile), oyster (proved *oy*), tomato, *to* (fertile). Among 387 daughters given breeding test, in general producing over 30 sons each, 16 proved heterozygous for mutant traits—eye colors: oyster, *oy* (fertile), scarlet, *st* (fertile), vermilion, *vm* (fertile) (4 + : 5 mutants), vermilion (proved *vm*), garnet, *ga* (fertile) (58 + : 6 mutants), scarlet-4 (fertile) (14 + : 19 mutants, scarlet-5 (fertile) (50 + : 58 mutants), light scarlet (28 + : 9 mutants found dead); body colors: blue-1 (19 + : 29 mutants found dead), blue-2 (sterile) (1 + : 3 mutants), blue-black (sterile) (124 + : 57 mutants), greenish-blue (sterile) (33 + : 23 mutants), purple (sterile) (15 + : 12 mutants); wings: shredded (sterile) (37 + : 20 mutants); legs: short-1 (sterile) and short-2 (sterile). No mutations have been found among many thousands examined from untreated stock. The 22 from the treated were distributed irrespective of dose or of age at time of treatment.

Seminar, August 16, 1949, Marine Biological Laboratories

The Development of Menidia-fundulus Hybrids. James M. Moulton.

Menidia beryllina beryllina ♂ × *Fundulus heteroclitus* ♀ hybrids survive until the hatching of controls, as reported and briefly described by Clark and Moulton (*Copeia*, 1949, No. 2), but have thus far failed to hatch. The reciprocal cross advances only to the yolk-plug stage. Both crosses of the other *Menidia* race found in the Woods Hole region, *Menidia menidia notata*, with *Fundulus heteroclitus* survive only to the yolk-plug as shown by Moenkhaus (1904, *Am. J. Anat.*, 26–65). The possibility is suggested that *M. b. beryllina* may have been the form used by J. Loeb in his hybridization experiments (1912, *J. Morph.*, 23, 1–15; 1915, *Biol. Bull.*, 29, 50–67).

Naming the hybrid embryo according to the egg used, the *M. b. beryllina* hybrid begins to lag in its developmental rate as compared with that of controls by the fifth cleavage—about three and a quarter hours at 18.5 degrees C. The *Fundulus* hybrid shows the first signs of such a lag at about 16 hours, when the expanding blastula is present in the controls. Subsequent to these stages it is rather difficult to distinguish between the effects of developmental lag and the origin of other anomalies in producing differences between hybrids and controls.

Among the anomalies observed in the hybrids are failure in yolk resorption which may be associated with other irregularities, incomplete development of the circulatory system and a rather amorphous nature to the embryo itself. Common anomalies involve the eyes. Some batches of hybrids have demonstrated such anomalies in nearly 100% of the embryos, the defects ranging from an approach of the optical organs to each other, through a perfect cyclopian condition, to a complete absence of optical structures so far as visible externally.

ERRATA

THE BIOLOGICAL BULLETIN, VOLUME 97, NO. 2, P. 260

Extrusion of jelly by eggs of Nereis limbata under electrical stimulus. W. J. V. OSTERHOUT.

In the first paragraph of the abstract noted above, "17 milliamperes" should read "170 milliamperes" and "much larger direct currents" should read "comparable direct currents."

In the third paragraph, "17 milliamperes" should read "170 milliamperes."

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